THE JOURNAL O
GENERAL PHYSIOL

Founded by Jacques Loeb

EDITORS
W. J. CROZIER JOHN H. NORTHROP
W. J. V. OSTERHOUT

VOLUME 14, No. 2 NOVEMBER 20, 1930

PUBLISHED BIMONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter November 25, 1910, at the Post Office at Baltimore, Md., under the act of March 3, 1879.

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ELECTROKINETIC PHENOMENA. III

The "Isoelectric Point" of Normal and Sensitized Mammalian Erythrocytes

By HAROLD A. ABRAMSON

(From the Laboratory of General Physiology, Harvard University, Cambridge)

(Accepted for publication, July 24, 1930)

I

INTRODUCTION

Kozawa (1) observed in 1914 that the addition of sufficient acid to mammalian red blood cells brought them to an isoelectric point at a pH which was characteristic for the cells of each of the animals studied. The amount of acid needed, however, was not constant for any particular animal, but decreased with the time the cells were permitted to stand in contact with the acid. It is evident, therefore, from these early experiments that time as well as acidity was influential in producing a reversal of sign of charge. These facts have not hindered a more or less general acceptance of the isoelectric point of red blood cells as a physical constant, apparently in much the same sense that it exists for the proteins. The reversal by hydrogen ion of the sign of charge of surfaces coated with proteins like egg albumin, and of globulin particles, occurs independent of time, with equilibrium, as far as our present technic permits, occurring instantly and remaining constant. The isoelectric point of a system is not significant in a simple sense unless it remains constant with time. If it does not remain constant, the variation points to a change in the surface or substance investigated and the "isoelectric point" does not represent a physical constant characteristic of the system. If the point of equilibrium is unrelated to the original system, except in a secondary way, the isoelectric point determined may be the resultant of many forces whose resolution can hardly be determined by the simple determination of reversal of sign of charge.

Coulter (2) (1920) studied the migration of sheep's red blood cells washed with saline solution and suspended in a saccharose solution made acid with HCl, acetate, or phosphate buffers. He used a macroscopic method which involved long experimental periods before readings of mobility could be made. Coulter concluded from his data:

". . . . that the direction and rate of movement in the electric field of both normal and sensitized red blood cells is a function of the hydrogen ion concentration. At concentrations less than pH 4.6 the charge carried is negative and increases in amount with the alkalinity; pH 4.6 represents the isoelectric point; at concentrations greater than pH 4.6 the charge carried is positive and increases in amount with the acidity."

"A comparison of the two curves[1] shows that on the alkaline side of the isoelectric point the charge of normal cells is greater and increases more rapidly with alkalinity than the charge of sensitized cells."

Shortly after the work of Coulter it was found by Eggerth (3) that the migration velocities of *B. coli* suspended in buffer mixtures commonly used for acid agglutination underwent a change in mobility, the organisms becoming less negative. He concluded that these changes were due to the extraction of a protein which was combined at the interface of organism and medium. In a second paper Eggerth (using a microscopic method to determine mobility) pointed out that similar changes occurred in erythrocyte suspensions.

Eggerth states, on the one hand:

"Human and sheep erythrocytes when placed in 0.01 N phthalate buffer solutions at reactions more acid than pH 5.2 undergo a progressive change in potential, becoming less negative or more electropositive. This change usually occurs within two hours at ordinary room temperatures. . . . This change is primarily due to the liberation of hemoglobin from the cells."

On the other hand, after thus showing that red cells were destroyed in acid solutions and that their mobilities varied with time, he states:

"The isoelectric point of erythrocytes in the absence of salt or in the presence of salts having both ions monovalent occurs at pH 4.7. This confirms the observations of Coulter."

---

[1] Curves for normal and sensitized cells.

Netter (4) has made a careful investigation of the electrophoretic mobilities of horse and ox cells in acid solutions to determine the isoelectric points. The isoelectric point of washed and unwashed horse red cells was at pH 4.7 in M/40 acetate. Washed ox cells were found to be isoelectric at pH 4.3 in M/40 acetate.

These data of Netter are concordant with those of Eggerth, not only in the existence of an isoelectric point for the red cells investigated but also in the fact that near the isoelectric point some time passed before "equilibrium" was obtained. He states:

"Das Potential stellt sich nach Uebertragen in die Loesung im allgemein schnell ein und bleibt constant, nur in der Gegend des isoelektrischen Punktes is das endgultiges Potential erst nach *2* Stunden spätestens erreicht; ***alle Messungen wurden daher nicht vor 2 Stunden nach dem Ansetzen der Suspensionen begonnen.***"

Netter cites further the data of Eggerth, mentioning the effect of the products of red cell destruction.

Eagle (5) (1930) states:

"Similarly, sensitization changes the cataphoretic potential of red cells; however, according to Coulter and confirmed by us, the cataphoretic isoelectric point remains at its *normal* value (pH 4.7)"

(the italics are ours). Further on this author writes:

"Red cells[2] both normal and sensitized have a minimum velocity at about pH 4.7: in more acid reaction there is hemolysis, with currents making readings impossible."

Fig. 1 summarizes graphically some of the pertinent results of Coulter, Eggerth, Netter and Eagle. All of the data of these authors are early or late *equilibrium* values of the isoelectric point obtained in *acid* solutions incidental to the presence of the products of hemolysis.

The existence of an isoelectric point for the mammalian red cell will be here more closely scrutinized in the light of these previous investigations and of our own. It will be shown that it is, at present, more in keeping with experiment to believe that normal red cells have no known values of pH for their isoelectric points, when suspended in uni-univalent electrolytes. While reversal of sign of charge may occur,

[2] The animal from which the red cells were obtained is not given.

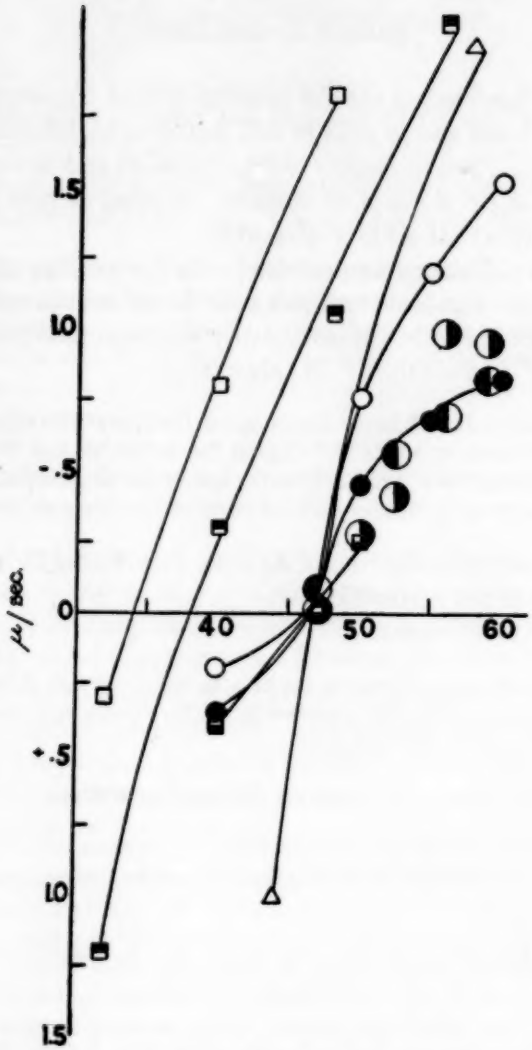

FIG. 1. The relationship between pH and electric mobility according to various authors:

○ Coulter; normal cells (relative units).
● Coulter; sensitized cells (relative units).
□ Eggerth; zero time
▣ Eggerth; 1.5 hours
▬ Eggerth; rabbit cells in 0.01 N NaCl.
} Human cells in saccharose buffered by 0.01 phthalate.
△ Netter; washed horse red cells in sugar buffered by N/40 acetate.
◑ Eagle; normal red cells in NaCl solution buffered by acetate (relative units).
◐ Eagle; sensitized red cells.

For further description, see text.

it will be shown that its demonstration is most unlikely with intact, normal human and sheep red blood cells.[3]

II

Methods

The measurements were performed in the modification of the Northrop-Kunitz cell described previously (6). Measurements in acid solutions were usually made only at one level (0.2) in the cell, since destruction of cells occurred in acid solutions so rapidly that further measurements were inconvenient. At the level, 0.2, the speed of the particle relative to the liquid is measured.

All measurements were made within two minutes of the time that acid was added to the system.

The description of the experimental procedure is best given with each experiment, in the next section.

The red cell suspensions were buffered sufficiently to prevent the occurrence of significant changes in pH due to hemolysis. No experiments with saccharose or other sugar were performed, for the following reason. Change in ionic strength is known to influence greatly the solubility and state of proteins. More normal conditions may be obtained for proteins existing at a phase boundary by not varying ionic concentration or species unnecessarily. If the ionic strength is varied it is evident that a new variable is introduced.

Hydrogen ion activity was determined by the quinhydrone electrode. All measurements are referred to pH 4.62 as the pH of Michaelis acetate standard.

III

The Isoelectric Point of Normal and Sensitized Cells

Experiment I.—Unwashed Human Red Blood Cells. Human blood was drawn up into the stem of a white blood cell pipette from a pricked finger and mixed directly with 100 cc. of 0.85 per cent sodium chloride solution buffered by N/50 sodium acetate-acetic acid solutions. Measurements of mobility were made within 2 minutes of preparation of the suspension.

Note that reversal of sign of charge did not occur; nor was there an appreciable decrease in mobility with decrease of pH. The fact that the mobility at pH 3.9 was so close to that observed at pH 5.7, where practically no destruction of red cells occurs within the short interval,

[3] Preliminary data have also been obtained for rabbit red cells. The conclusions are similar.

indicates that these mobilities are related more to the normal chemical constitution of the red cell than to complex phase boundary conditions occurring in acid media.

Experiment II.—Washed Human Cells. 5 cc. of human blood were poured into 25 cc. of 0.85 per cent NaCl and centrifuged. The cells were washed 6 times with 10 times their volume of 0.85 per cent NaCl containing N/100 sodium acetate. Measurements of mobility were made within 2 minutes after addition of acetic acid.

Table II confirms that data found in Table I.

TABLE I

pH	V
	μ/sec/volt/cm.
5.7	−1.15
4.6	−1.03
4.0	−0.97
3.9	−1.02

TABLE II

pH	V μ/sec/volt/cm.
5.6	−1.05
4.1	−1.10
3.6	−1.04
3.55	−0.92

Experiment III.—Washed Sheep Cells. Fresh defibrinated sheep cells which had been in the ice chest for 12 hours[4] were washed 8 times with about 10 times their volume of 0.85 per cent sodium chloride solution containing N/50 sodium acetate. The acetate was added to keep the suspension alkaline. Suspensions of red cells were made up as in Experiment I and measurements were made within 2 minutes.

Because destruction of cells occurs with such rapidity in acid solutions, the slight diminution in mobility described in Table III cannot be unequivocally correlated with decreasing pH. The possibility

[4] No appreciable change in mobility occurs if horse red cells are kept in serum in the ice chest as long as 3 days.

remains that this slight but definite change may be due to adsorption of the products of erythrocyte destruction. This will be subsequently discussed in detail.

Experiment IV (A).—Sheep Cells in the Presence of Rabbit Serum. Sheep cells of Experiment III[5] were added to 50 cc. of 0.85 per cent sodium chloride containing, in addition, N/50 sodium acetate, and 1 cc. of washed[6] normal rabbit serum which had been heated to destroy complement. The sheep cells were allowed to

TABLE III

pH	V μ/sec/volt/cm.	Remarks
6.45	−1.16	In these acid solutions hemolysis was very rapid and at times complete within 2½ minutes
6.26	−1.03	
5.91	−1.12	
4.73	−1.01	
4.30	−0.98	
3.72	−0.97	
3.62	−0.94	
3.60	−0.88	

TABLE IV

pH	V μ/sec/volt/cm.	Remarks
6.18	−1.14	Control: No serum; pH 3.51, V = −.75 μ/sec/volt/cm.
4.25	−0.97	
3.90	−0.56	
3.67	±	

remain in contact with this solution for 15 minutes. Measurements of mobility were made within 2 minutes after addition of acid. See data in Table IV.

Table IV demonstrates that above pH 4.2 the presence of rabbit serum protein does not appreciably influence sheep red cell mobility. This is in harmony with our previous findings that human cells sus-

[5] Twelve hours later and washed twice with salt-acetate.

[6] This refers to a preliminary treatment of the "normal" rabbit serum with rabbit red cells to remove any amboceptor. This treatment does not remove an appreciable quantity of serum protein.

pended in isotonic media containing 1.4 per cent of rabbit serum retain their surface integrity and migrate about twice as quickly as rabbit cells suspended in the same medium (7). The interpretation of the fact that red cells are isoelectric at about pH 3.6 is difficult. Since this change in mobility occurs in a region where red cell destruction is extremely rapid, it seems reasonable to conclude that the drop in

TABLE V

pH	V μ/sec/volt/cm.	Remarks	
		Controls pH	Without serum V μ/sec/volt/cm.
5.50	−1.00	5.55	−1.04
4.65	−1.00	4.65	−0.91
4.60	−0.95	3.54	−0.79
4.10	−0.53		
3.74	negative		
3.64	positive		

TABLE VI

pH	V μ/sec/volt/cm.	Remarks	
		Controls pH	No serum V μ/sec/volt/cm.
6.5	−0.58	4.72	−1.04
4.97	−0.60	3.5	−0.89
4.70	−0.45		
4.0	−0.43		
3.6	±		

mobility and attainment of an isoelectric state is determined by the presence of abnormal red cell surfaces as well as by the presence of serum protein. We believe, therefore, that the isoelectric point noted has no more significance than that which may be attached to those red cell systems described in the introduction.

Experiment IV (B).—Sheep Cells in Presence of Rabbit Serum. Experiment repeated in N/100 acetate buffer. See data in Table V.

The discussion following Table IV applies here.

Experiment V (A).—Sensitized Sheep Cells. The sheep red cells used in IV (B) were kept in the ice chest for 6 days. At the end of this time they were washed 8 times with about 10 times their volume of 0.85 per cent sodium chloride containing N/50 sodium acetate. A very dilute suspension of the cells (about 1:2000) was permitted to remain about 15 minutes in contact with 24 cc. of physiological saline containing 1 cc. of an inactivated rabbit anti-sheep serum. At the end of this period a suitable mixture of electrolytes was added to make conditions identical with part (b) of this section. Measurements were completed within two minutes. See data in Table VI.

Experiment V (B).—Sensitized Sheep Cells. Experiment V (a) was repeated in N/100 acetate buffer. See data in Table VII.

TABLE VII

pH	V μ/sec/volt/cm.
5.5	−0.57
4.35	−0.60
4.1	−0.40
3.9	−0.28
3.8	negative
3.7	±
3.6	+0.24

The data of Tables VI and VII demonstrate the well known effect of specific immune serum on red cell mobility. In one sense they confirm Coulter's statement that the isoelectric point of sensitized cells is not perceptibly different from that of normal cells. In view, however, of the fact that an excess of serum protein is present, and furthermore that protein of the serum alone is capable of inducing an isoelectric state near the pH noted, we feel we must ascribe the similarity found here for the isoelectric point of normal and sensitized cells to the same effect, possibly an injurious one, that has been described as occurring in solutions as acid as these. Indeed, the wide differences in mobility between normal and sensitized cells in the zones where injury to the cell surfaces is not marked, make it more likely that if isoelectric points with regard to hydrogen ion activity could be determined without red cell destruction, these points would be different.

What effect more powerful immune sera would have on the mobility is unknown for these conditions (See Fig. 2).

IV

The Adsorption of Gelatin by Red Cells

It has been previously remarked on several occasions that horse red cells in (alkaline) serum gelatin gels do not adsorb gelatin in the same

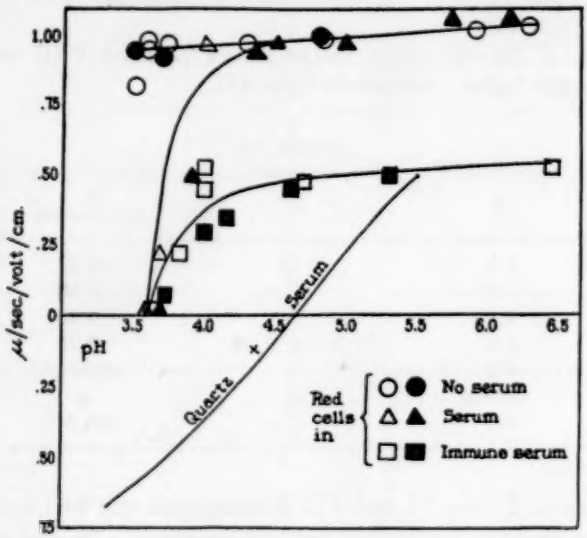

FIG. 2. A summary of the type of data obtained here for the electrophoretic mobilities of sheep red cells in 0.85 per cent NaCl buffered by acetate. Note that in the absence of serum under the experimental conditions noted in the text, the slope of the straight line describing the relationship between mobility and pH is very small. Serum has no demonstrable effect up to about pH 4.3. Immune serum changes the surface notably even above pH 4.3. Compare with Fig. 1. Quartz particles adsorb protein of the serum in characteristic fashion. (See: Abramson, H. A., *J. Gen. Physiol.*, 1929, **13**, 169.)

way that quartz particles do (8). That is, in just the same way that horse red cells preserve their surface integrity in the presence of serum, so do washed red cells keep their specific electrophoretic mobilities in the presence of gelatin in phosphate buffer at pH 7.35.

We have seen in the preceding section that adsorption of some serum protein probably does occur in acid solutions. It was of interest therefore to observe the mobilities of washed red cells that had been in contact with gelatin, and studied in acid solutions in the presence of gelatin.

Experiment VI (A).—Washed Sheep Cells (Table VIII).

TABLE VIII

pH	V μ/sec/volt/cm.	Remarks
3.61	−0.87	Cells of Experiment II (b) in 0.2 per cent gelatin

Experiment VI (B).—Washed Human Cells (Table IX).

TABLE IX

pH	V μ/sec/volt/cm.	Remarks
5.9	−1.00	Cells of Experiment III kept 24 hours on ice, rewashed, and studied in 0.2 per cent gelatin
4.33	−1.06	
3.62	−0.92	
Control, quartz		
3.6	+0.40 approx.	
5.7	−0.25 approx.	

Experiment VI (C).—Washed Sheep Cells (Table X).

TABLE X

pH	V μ/sec/volt/cm.	Remarks
4.00	−0.93	Cells of Experiment IV (b) suspended in 0.2 per cent gelatin for 10 minutes and then examined
3.76	−0.75	
3.67	−0.65	

The data in Tables VIII, IX, and X indicate that even though red cells in acid solutions may react with certain components of a medium containing normal serum, a similar reaction does not occur with gela-

tin. One is led to suspect that the reaction with the protein of the serum is not exactly as non-specific as the adsorption of serum protein by quartz. If so, the red cells should have adsorbed gelatin. Experiment VI (B) was made particularly striking by studying simultaneously quartz particles and red cells in the presence of gelatin. In these systems the red cells migrated to the anode and the quartz particles, because of their adsorbed protein film, migrated in the opposite direction.

It must not be thought that reversal of the sign of charge of red cells did not occur in the acid solutions. It did so repeatedly, but always after the 2-minute period of contact with the salt solution when the products of hemolysis were sufficiently concentrated to be an important factor. In *no case* was a positively charged red blood cell seen in acid solutions during the 2-minute period of observations.

It is of interest to describe how this reversal of sign of charge may occur. In Table X the cells suspended in the buffer at pH 3.67 were observed after the end of the 2-minute period during the very rapid hemolysis which occurs at this pH. Red cells which were seen migrating rapidly to the anode seemed to lose their hemoglobin suddenly and the pale "ghost" reverse its sign of charge migrating to the cathode.

V

The Reaction of Sheep Cells with Normal and Immune Sera in Phosphate Buffer[7]

An extensive series of measurements was made to investigate the effect of normal and immune rabbit sera on sheep cells We shall not give the data in detail, for these experiments in general merely confirm what has hitherto been known qualitatively. They are of interest because of the precision which has been obtained in 0.85 per cent NaCl buffered by M/150 phosphate of initial pH 7.35, the medium in which all observations of mobility have been made, and because of the associated studies of hemolysis made in conjunction with changes in mobility. In the experiments described in general in Fig. 3, five different immune sera and four normal sera were employed. Curve 1 in Fig. 3

[7] When studying the effect of sera on cells, at least 1 hour's time elapsed between preparation of suspension and measurement.

was obtained by studying washed sheep cells in normal inactivated rabbit serum. It is evident that no change in mobility occurs up to about 1:50 of serum. Curve 2 has also been obtained with normal serum from another rabbit. At low serum dilution a definite lowering of electrophoretic mobility occurred. That this change was real, was evidenced by the fact that after addition of more sheep cells to the suspension, cells having two different mobilities were present. Curve 3 is a typical curve obtained with inactivated anti-sheep serum. Approximately the same value of mobility was obtained for all the

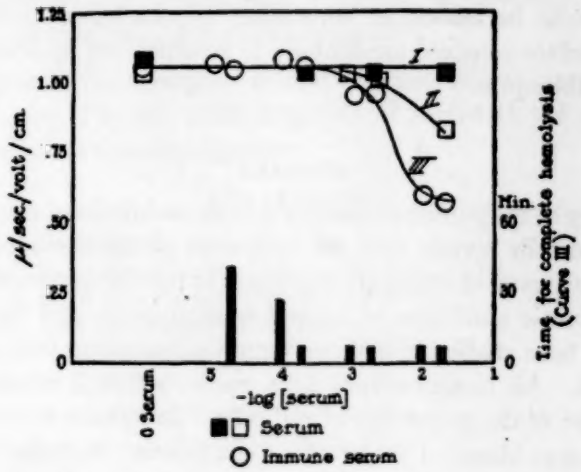

FIG. 3. See Part V of text. The upright black rectangles represent hemolysis times.

immune sera. Whether this is the lowest mobility obtainable cannot be decided upon from the shape of the curve, for the possibility exists that a more powerful anti-serum may be obtained than that employed.

The cells with which Curve 3 had been obtained were studied with a suitable dilution of complement to ascertain the relationship between the lowering of mobility and susceptibility to hemolysis by complement. It is to be noted that complete hemolysis on the addition of complement (0.03 cc. guinea pig serum to 1 cc. of a dilute red cell suspension) occurred in all except the highest dilution of serum, much before any perceptible change in mobility occurs. In other words,

sensitization to hemolysis of the red cell surface occurs without any change in mobility. In fact, hemolysis was just as rapid in the 1:5000 dilution as in the lower ones. At this dilution, as is apparent from the figure, there is no change in mobility, but hemolysis is rapid and complete. Since the mobility of the cell is in general determined by its surface constitution, very few active groups of the cell surface are changed for preparation of the cell for hemolysis incidental to the presence of complement. The lowering of the electrophoretic mobility does indicate a notable change in the surface make-up of the cell. As far as hemolysis requirements are concerned this change is secondary and must be associated with other immunological properties of the cell surface acquired incidentally to sensitization by a large quantity of amboceptor.

VI

SUMMARY

A survey of the published electrophoretic mobilities of certain mammalian red cells reveals that the isoelectric points accorded to these cells are the result of equilibria incidental to red cell destruction. The electrophoretic mobilities of normal washed sheep and human cells have now been studied in 0.85 per cent NaCl solutions from about pH 3.6 to 7.4. All measurements were made within 2 minutes of the preparation of the suspension of red cells. In no case was reversal of sign of charge observed under these conditions. Reversal of sign of charge occurred only after sufficient time had elapsed to permit sufficient adsorption of the products of red cell destruction. There is little change in mobility as the pH of the medium is decreased. Reversal of sign of charge does occur in the presence of normal and immune (anti-sheep) rabbit sera. The isoelectric point determined under these conditions does not appear to be connected specifically with the immune body but is perhaps associated with phenomena incidental to red cell destruction and the presence of serum. The characteristic lowering of mobility by amboceptor occurs, however, from pH 4.0 to pH 7.4. The curves of mobility plotted against pH for normal and for immune sera support the viewpoint that the identity of the isoelectric points for normal and sensitized sheep cells is not primarily concerned with the immune reaction. It is most unlikely that an "albu-

min" or a "globulin" surface covers red cells with a complete protein film. Although serum protein reacts with red cells in acid solutions, this is not demonstrable for gelatin. The lowering of mobility usually ascribed to anti-sheep rabbit serum may also occur, but to a lesser degree, in normal rabbit serum. This diminution of mobility is not, in the first place, associated with sensitization to hemolysis induced by complement. This supports the view that only a very small part of the red cell surface need be changed in order to obtain complete hemolysis in the presence of complement.

I am indebted to Professor Wm. J. Crozier for reading the manuscript and making many acceptable suggestions.

Addendum.—The viewpoint of McCutcheon, Mudd, Strumia, and Lucké (*J. Gen. Physiol.*, 1930, **13**, 669) in regard to the isoelectric point of cells has recently appeared.

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FACTORS INFLUENCING THE RESPIRATION OF ERYTHROCYTES

I. Primitive Avian Erythrocytes

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(Accepted for publication, July 28, 1930)

INTRODUCTION

The function of the nucleus as the principal seat of the oxidative processes in the cell was first suggested by the investigations of Spitzer (1) upon the location of oxidizing enzymes. These observations were given considerable support by Lillie (2), who studied the problem with the aid of dyes that underwent color changes upon oxidation. He found that "the coloured oxidation products are seen to be deposited chiefly in and about the nucleus, especially at the surface of contact between nucleus and cytoplasm." These studies were followed up by Warburg (3) in a series of observations upon the comparative rates of reduction of the hemoglobin in the blood of mammals and birds, incubated for some hours in sealed vessels. He found that there existed a very marked difference between the two: the non-nucleated cells of the mammals had a comparatively small consumption of oxygen, whilst the nucleated ones in avian blood possessed a vigorous oxidative metabolism. He further showed that in both mammals and birds the oxidative metabolism proceeded at a much greater rate in blood which contained many young cells staining in a basophilic manner with methylene blue. In fact it appeared doubtful whether the fully developed normally staining mammalian red blood cells consumed oxygen to any appreciable extent. The whole of the slight oxygen consumption of the normal blood of adult mammals would seem to be due to the small proportion of young reticulated and basophilic cells ordinarily in the circulation.

The very large oxygen consumption of avian blood, especially after

hemorrhage when the proportion of primitive red cells in the circulation was high, led Warburg to a study of the factors which influence its metabolism. Many of the experiments were concerned with those factors that reduce or inhibit oxidation, and culminated in his well known theory of the importance of the part that structural integrity plays in the processes of cell oxidations (4).

The following experiments were carried out with the intention of determining rather more fully the effects that certain changes in environment produce upon the metabolism of the primitive avian red cells.

Methods

The blood used in all the experiments was removed by cardiac puncture from fowls made anemic by intraperitoneal injections of phenylhydrazine hydrochloride. 10 cc. of a one half per cent solution in normal physiological saline was given to each bird. The blood was used from the third to the seventh day following the injection, and the removal, either daily or every second day, of 30 cc. required for each experiment assisted in maintaining the anemia for this period. Immediately the blood was drawn it was placed in small bottles and shaken with glass beads until defibrinated. It was then filtered through a small wad of cotton wool to remove the clot and was then ready for use.

The blood was generally removed immediately before the experiment was set up. Occasionally, when kept for a few hours, it was placed in the cold room. Harrop (5) has shown that at low temperatures the viability of the cells is well maintained. In his experiments there was only a small reduction in the oxygen consumption of cells incubated at 38°C., after having been kept for 24 hours at 7°C., as compared with cells from the same blood which were incubated as soon as they were removed from the animal. My own observations are in agreement with his finding.

The oxygen consumption of the blood was determined in the respiration apparatus devised by Barcroft and modified by Warburg (6). 2 cc. of blood or of cell suspension were invariably used for the estimations. The oxygen consumption was determined at a temperature of 37°C.

The apparatus proved very satisfactory for this purpose; the oxygen consumption of this quantity of blood in flasks of about 15 cc. capacity usually causing a movement of the fluid meniscus in the manometer tube of some 10 to 20 cm. in the 90 minutes duration of the experiment. After the blood was placed in the apparatus the flasks were shaken about eighty times a minute for half an hour in the thermostat tank for the purpose of raising the temperature of the blood to 37°C. and also of fully oxygenating the red cells. The carbon dioxide given off was absorbed by normal sodium hydroxide placed in the small central cups in the flasks.

The possible sources of error in determining the oxygen consumption of blood have been discussed by Morawitz (7), by Warburg (3), and by Harrop (5) and

consequently need little consideration here. The duration of the experiments carried out by these investigators frequently extended over a period of many hours and consequently necessitated an aseptic technique. These observations were completed within 2 hours of the commencement of incubation and consequently no especial precautions were taken to ensure absolute sterility.

The question of the applicability of the Warburg method to this type of experiment requires more consideration. Two possible sources of error in the determinations need to be discussed. First, the evolution of carbon dioxide from the blood might possibly have some effect in causing the hemoglobin to take up small quantities of oxygen by a change in its affinity and so giving a spurious appearance of true oxidation by the red cells themselves. Secondly, all the hemoglobin may not be present in the form of oxyhemoglobin by the time the actual metabolism measurements are started. That no serious error from either of these causes occurs is shown by experiments upon the blood of normal rabbits containing only a small number of reticulocytes. Such blood, after the usual equilibration period of 30 minutes, shows a practically negligible oxygen consumption, indicating that by that time all the hemoglobin in the cells has become completely oxygenated. Further, this small consumption of oxygen continued with only slightly diminishing intensity for the ensuing 90 minutes, the duration of the experiment.

The degree of accuracy of the actual determinations may be judged from the following measurements of the oxygen consumption of the same samples of blood carried out simultaneously in six manometer tubes:

Tube Numbers	1	2	3	4	5	6
	C.mm. oxygen consumed per cc. blood per hour					
Expt. 1	123.8	122.2	122.4	122.0	124.0	123.6
Expt. 2	64.0	63.5	64.6	63.6	64.3	63.5

I

Metabolism in Phenylhydrazine Anemia

Phenylhydrazine has been extensively used in the production of experimental anemias in animals. It was first made use of in birds by Hirsch and Edel (8). Later it was employed by Kasarinoff (9) in his study of the primitive blood cells in fowls and his paper contains several excellent illustrations of the early red cell forms. When fowls are injected intraperitoneally with phenylhydrazine in medium doses, (20 mg. per kilo) in the course of a few minutes there is a striking

change in their appearance. Their combs assume a dusky brown color, and if blood be drawn at this stage it exhibits the same discoloration. This chocolate brown color of the blood persists for 48 to 72 hours before finally passing off, its termination being roughly coincident with the point of greatest severity in the anemia. The recovery from the anemia takes place fairly rapidly at first and then more slowly, being characterized by somewhat considerable oscillations in the red cell counts, a condition previously observed in the phenylhydrazine anemia of rabbits by Morawitz (7).

TABLE I

Fowl No.	Days after injection	Red cell count in millions per c.mm.	Oxygen consumption		
			C.mm. per cc. blood per hour	C.mm. per billion red cells per hour	Ratio of anemic to normal blood
24	0	3.27	29.7	9.1	—
	2	1.12	118.2	105.4	11.6
	4	1.98	163.8	82.8	9.1
	6	2.34	72.3	30.9	3.4
	8	1.76	29.8	15.9	1.8
	10	1.98	30.2	15.2	1.7
	12	1.80	31.1	17.3	1.9
25	0	3.53	26.9	7.6	—
	2	1.49	129.1	86.7	11.4
	4	2.27	164.2	72.3	9.5
	6	2.37	64.8	27.3	3.6
	8	2.27	37.3	14.5	1.9
	10	1.82	24.4	13.4	1.7
	12	2.23	37.7	16.9	2.2

Both fowls were injected with phenylhydrazine hydrochloride 0.5 per cent solution in saline (20 mg. per kilo) immediately after the blood was taken for the oxygen determinations on the first day of the experiment.

In this experiment observations were made both on the red cell counts and on the oxygen consumption of the blood. Two fowls were injected intraperitoneally with a one half per cent solution of phenylhydrazine in normal physiological saline (20 mg. per kilo). Immediately previous to the injection 12 cc. of blood were removed from each bird by cardiac puncture and its oxygen consumption determined. These latter were 9.1 and 7.6 c.mm. of oxygen per billion (one thousand

million) red cells per hour. Every second day afterwards 12 cc. of blood were similarly removed and the oxygen consumption and red cell count determined. The results are found in Table I and in Fig. 1.

The onset of the anemia was accompanied by a striking rise in the oxygen consumption of the cells, which at its peak attained a value of more than ten times its initial figure. At the same time the morpho-

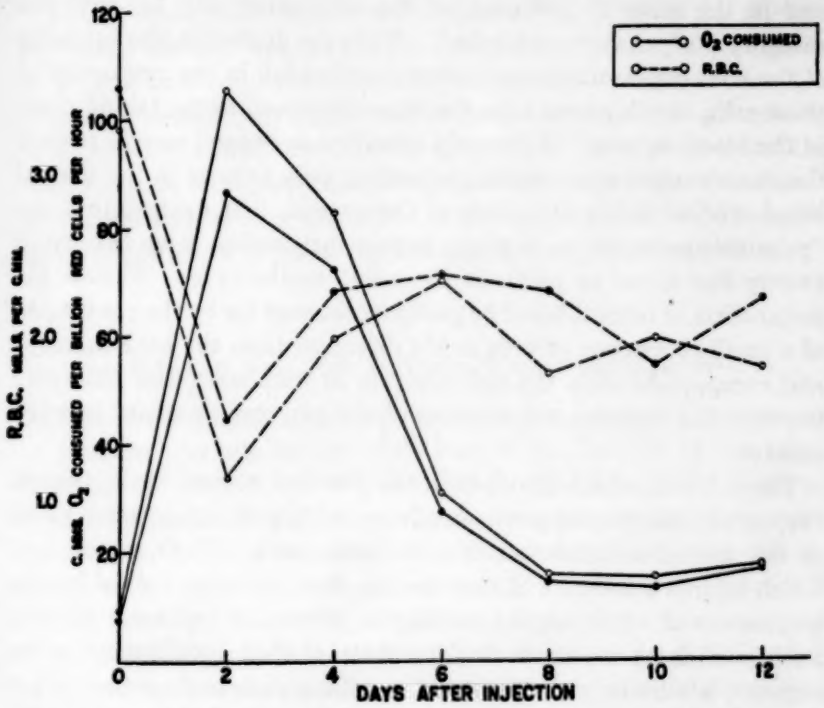

FIG. 1. Showing the oxygen consumption of avian red cells at various stages of an anemia produced by injection of phenylhydrazine.

logical appearances of the blood showed the marked changes described by Kasarinoff (9). Large numbers of the primitive hematoblasts were present together with many cells at all stages in the development of erythrocytes.

The enumeration of the various forms of primitive cells proved difficult on account of the many transitional varieties, and no attempt at division into specific categories was made other than into two large

groups: erythrocytes of normal appearance, and "primitive red cells" inclusive of all cells from the hematoblast to the large hemoglobin-containing cells still exhibiting vitally staining reticulum. Differential counts carried out in this way upon blood films, stained vitally with brilliant cresol blue followed by Wright's stain, showed that, at the peak of metabolism on the second day, in one blood 40 per cent and in the other 38 per cent, of the circulating cells fell into the category of "primitive red cells." With the decline in the intensity of the metabolism there was a corresponding fall in the proportion of these cells, development into the more advanced forms taking place in the blood stream. If the cells classified as normal merely possess the same oxygen consumption as similar cells present in the normal blood studied before the onset of the anemia, it is evident that the "primitive red cells" as a group have a metabolism some twenty to twenty-five times as great as the adult erythrocytes. Should the metabolism of normal blood be partly accounted for by the circulation of a small proportion of cells newly delivered from the bone marrow, and comparable with the reticulocytes of mammals, the difference between the oxygen consumptions of the two groups would be even greater.

The red cells which circulate in the blood of normal birds, though they retain their nuclei, are in an advanced state of degeneration so far as the normal cellular activities are concerned. Their respiration, which affords a measure of their metabolism, is low as judged by the respiration of other organs, while the process of pyknosis of their nuclei, which takes place in the later states of their development in the marrow, is indistinguishable from that taking place in the cells of other degenerating tissues. On the other hand the "primitive red cells" in the process of their transformation in the circulation into the normal adult erythrocytes consume oxygen at a rate of approximately 0.5 to 1.0 c.mm. per mg. per hour. While such an oxygen consumption is still somewhat smaller than that observed by Barcroft and Shore (10) in the cells of the liver of the cat, and by Usui (11) in those of the liver of the mouse, it must be recalled that the erythrocyte has included in its cytoplasm a relatively large quantity of oxyhemoglobin which has no oxygen consumption in the ordinarily accepted sense of that term. If allowance be made for this cellular inclusion the metab-

olism of the "primitive red cells" of the fowl becomes comparable with that of the cells of a parenchymatous organ like the liver. This relatively high metabolism, together with the resistance of the red cell to mechanical injury and its life in a fluid matrix of well determined composition, combines to render it a very suitable type of cell for the study of the influence of various factors upon cellular metabolism.

In the course of 8 days from the time of injection of the phenylhydrazine into the fowl the oxygen consumption of its blood had risen to a peak and returned nearly to its previous normal level. The persistent anemia was almost certainly due to the frequent removal of small quantities of blood for the purposes of the experiment. The continuous blood regeneration which just compensated for these losses was at the same time responsible for the somewhat elevated metabolism which was observed in the last few determinations made.

II

Effect of Variation in Tonicity upon Metabolism

The following experiments were carried out to determine how greatly metabolism was affected by variations in the tonicity of the saline solutions in which cells were suspended. The blood was obtained from fowls made anemic by an injection of phenylhydrazine. After centrifuging the blood for 5 minutes the supernatant serum was removed with a pipette and the cell suspension made up to its original volume with sodium chloride solutions of varying concentrations. Only one centrifugation was employed in order to minimize the inevitable injury to the cells caused by their being packed tightly together.

The observations are given in the following table:

Red cells in millions per c.mm.	NaCl: 0.6 per cent	0.75 per cent	0.85 per cent	0.95 per cent	1.10 per cent
	C.mm. O_2 consumed per billion red cells per hour				
2.05	32.2	32.0	32.0	33.2	32.8
1.33	36.7	37.9	39.7	39.6	32.8
0.92	41.7	42.6	44.8	44.6	41.1

While the effects of varying the tonicity of the saline solutions in which the cells are suspended are comparatively slight within the limits

employed in these experiments, it can be seen that the maximum metabolism occurs at some point between 0.85 per cent and 0.95 per cent of sodium chloride. Hematocrit observations made by the method described by Hirota (12) demonstrated that between these concentrations the size of the suspended red cells both in normal and anemic blood approximates most closely to that when they are present in serum.

Red cells in millions per c.mm.	Serum	0.75 per cent	0.85 per cent	0.90 per cent	0.95 per cent	1.05 per cent
	Hematocrit readings in per cent in NaCl solutions					
2.76	28.5	31.3	29.3		27.6	27.2
1.36	24.3			24.2		

These results are similar to those obtained by Ray (13) for the reticulocytes of the dog. He made observations on the oxygen consumption of these cells in the presence of M/12.5 solutions of sodium lactate to which sufficient sodium chloride was added to render them isotonic with the following concentrations of sodium chloride: 0.5, 0.7, 0.9, 1.1, 1.3 per cent. He found that the oxygen consumption attained its maximum in solutions isotonic with 0.9 per cent sodium chloride and that it declined rather more rapidly if the solutions were made hypertonic than if they were made hypotonic.

III

The Influence of Hydrogen Ion Activity upon Metabolism

The extent to which the hydrogen ion activity of the surrounding medium influenced metabolism in the avian red cell was determined in this series of experiments. The method employed here differed somewhat from that adopted in the other studies. The oxygen consumption of the cells was measured in their own serum to which certain known quantities of acid had been added. In order to examine whether any specific ionic effects were involved both hydrochloric and phosphoric acids were used.

To 2 cc. of blood from an anemic fowl 1 cc. of 0.87 per cent sodium chloride solution containing known amounts of these acids was added. The acidity of these saline solutions varied between N/200

TABLE II

The Effect of Variations in Hydrogen Ion Activity upon the Metabolism of Avian Red Cells

Concentrated (mols per l.) HCl in 0.87 per cent NaCl solution added to cells	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH
C.									
0.000	45.0	86	—	34.1	85	8.65	33.6	87	8.79
0.005	45.6	88	—	36.1	90	8.53			
0.010	48.9	94	—	37.6	94	8.03	36.4	94	8.02
0.020	52.1	100	—	40.0	100	7.80	38.7	100	7.90
0.030							38.8	100	7.64
0.040							35.2	91	7.44
0.050	45.3	87	—	35.2	88	7.27	28.7	74	7.22
0.100	27.1	52	—	19.0	48	5.85			
Red cell counts in millions.....	1.75			1.96			2.15		

Concentrated (mols per l.) H_3PO_4 in 0.87 per cent NaCl solutions added to cells	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH
C.									
0.000	29.6	92	8.34	47.4	91	8.26	45.0	84	8.46
0.005							45.9	86	8.19
0.010	30.8	96	8.04	51.9	100	8.10	49.8	93	8.08
0.015							53.4	100	7.88
0.020	32.1	100	7.65	52.0	100	7.53	53.5	100	7.62
0.025							45.1	84	7.30
0.030	24.9	78	7.28	40.3	77	7.18			
0.040	21.0	65	6.91	32.3	62	6.79			
0.050	18.6	58	6.66	29.8	57	6.56			
Red cell counts in millions.....	2.12			1.44			1.18		

* Oxygen consumption given in units of c.mm. O_2 per billion red cells per hour.

and N/10. 2 cc. of this cell suspension were used for the determination of its oxygen consumption. Immediately after the measurement of the metabolism was completed the suspension of cells was removed from the flasks of the Barcroft-Warburg manometers, rapidly centri-

fuged and the supernatant fluid removed. This separation of the cells was effected as quickly as possible to minimize any change in reaction of the solutions by the further metabolism of the red cells.

The reaction of the supernatant fluid was measured with the quinhydrone electrode. I am indebted to Dr. Arda Alden Green for checking a number of the more important points on the hydrogen

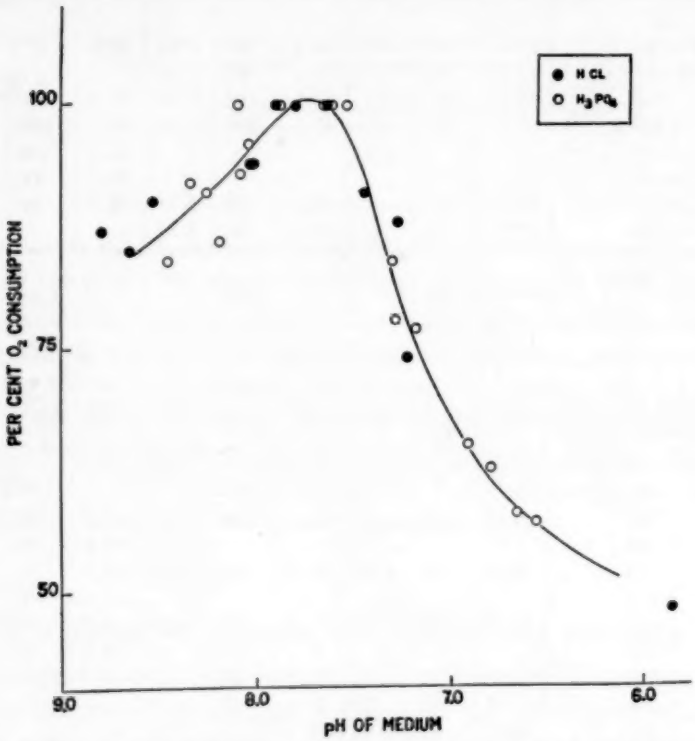

FIG. 2. Showing the influence of changes in hydrogen ion activity upon the oxygen consumption of primitive avian red cells.

electrode. The agreement between the two methods proved satisfactory up to a reaction of about pH 8.4.

The relative alkalinity of the sera, to which saline only was added, was due to the loss of a large part of its carbon dioxide in the first few minutes of shaking in the apparatus. This carbon dioxide was absorbed in the sodium hydroxide which was placed in the central cups

of the flask in order to combine with the carbon dioxide given off by the cells in the course of their metabolism.

The results are presented in Table II and in Fig. 2.

These experiments demonstrate that the addition of small quantities of acid to the serum in which the cells are suspended exercises a very considerable influence upon the oxygen consumption of the red cells themselves. This effect appears to be dependant upon the hydrogen ion concentration of the resulting mixture, it being immaterial whether this reaction be reached by the addition of phosphoric acid or hydrochloric acid. In the case of certain other acids their presence in concentrations necessary to bring about the range of reaction investigated here would most probably introduce specific ionic effects other than that from the dissociated hydrogen ion.

The range of variation studied was considerable: the lowest point being pH 5.85, and the highest pH 8.79. The maximum oxygen consumption took place at pH 7.75. In solutions of this reaction the contents of the corpuscles would probably have a considerably more acid reaction. Taylor (14) has estimated that at this reaction the corpuscular contents in goose blood have a pH value nearly 0.3 lower than the serum. On either side of this reaction the metabolism rapidly declined, until on the acid side at a pH of 6.5 it was only half of its value at the maximum. On the alkaline side the falling away did not seem to be so steep, but since the quinhydrone electrode is inaccurate at reactions more alkaline than pH 8.4 the figures in this range are less dependable. It is of interest that Fischer (15) in his investigations of the influence of hydrogen ion activity upon the growth and emigration of fibroblasts in tissue cultures found that these took place at their best between pH 7.4 and pH 7.8. He also observed that repeated cultivation in a medium on the alkaline side of pH 7.4 was much less injurious to the cells than cultivation in one more acid.

There exists the possibility that the hydrogen ion concentration of the media might undergo change during the course of the experiment and that the final determinations might give a false indication of the reaction at which the maximum oxygen consumption took place. Should the reaction change appreciably within the 90 minutes of the experiment it might be expected that, since the red cells are evidently

quite sensitive to such a change, the maximal metabolism in the last 45 minutes would be observed in a different observation flask from that of the experiment as a whole. This did not take place. In every case the flask of blood which registered the highest oxygen consumption for the whole experiment also registered the highest if the last 45 minutes were alone considered. It is consequently highly improbable that the reactions in the individual flasks varied appreciably in the period of observations.

IV

Effect of Variations of Glucose Concentrations upon Metabolism

A series of observations was made to determine the effect of the presence of small quantities of glucose upon the oxygen consumption of the red blood cells in the blood of anemic birds. The technical procedures were similar to those employed in the studies of the influence of tonicity. The blood from anemic fowls was defibrinated by shaking with glass beads and centrifuged for a period of 5 minutes in a series of graduated tubes. After the supernatant serum was removed the cell suspensions were restored to their original volumes by the addition of sodium chloride solutions (0.87 per cent) containing varying quantities of glucose. In the last experiment, where the glucose was added in rather high concentration, that of the sodium chloride was correspondingly reduced. It was found, by hematocrit determinations, that the cells both of normal and anemic fowls' blood had the same volume in 4.8 per cent glucose solutions as they had in their own serum and the concentrations of the sodium chloride present in the last experiment were adjusted upon this basis. No adjustment was made for variations in tonicity in the earlier experiments where lower concentrations of glucose were used. Previous observations have however shown that greater alterations in tonicity than were involved here have little influence upon the oxygen consumption of the cells, and this influence would in any case have been in the direction of reduction rather than acceleration of metabolism.

Table III and Fig. 3 show the results obtained.

It is evident that small increments in the concentration of the glucose present are accompanied by corresponding acceleration in the oxygen

consumption of the cells. With further increase in the amount of glucose, however, the metabolism was stimulated less and less until from 0.6 per cent to 1.2 per cent no change in the consumption of oxygen took place. The metabolism between these two points was steady at a level some 15 per cent above that in the corresponding glucose-free saline solution. We have not determined how far this condition of steady oxygen consumption continues with rising glucose concentration. Warburg (3) states that the blood cells in anemic geese consume the same quantity of oxygen in a 5 per cent glucose

TABLE III

C.mm. O_2 Consumed per Billion Red Cells Per Hour in Varying Concentrations of Glucose

Red cells in millions per c.mm.	0.91		1.66		1.98		1.98	
Concentration glucose	C.mm. O_2	Per cent of saline value	C.mm. O_2	Per cent of saline value	C.mm. O_2	Per cent of saline value	C.mm. O_2	Per cent of saline value
per cent								
nil	77.1	100	122.9	100	96.0	100	72.4	100
0.1	81.4	105	130.4	106	102.0	106		
0.2	83.6	108	132.0	107	102.0	106		
0.3	84.8	110	132.5	108	104.5	109	81.6	113
0.4			133.8	109				
0.5			134.3	110	106.5	111		
0.6							83.6	115
0.75					109.6	114		
0.9							83.4	115
1.2							83.4	115

solution as they do in Ringer's solution, which would imply a decline beyond a maximal plateau. I have previously carried out determinations (16) of the sugar concentration present in the serum of normal starved fowls and found it to be from 0.20 to 0.22 per cent. It is clear therefore that the maximal oxygen consumption of the red cells takes place at a glucose concentration several times that normally present in the serum.

The permeability of erythrocytes to glucose has been extensively studied, but so far as I have been able to find, in every case upon the normal fully developed cells and in nearly all cases upon those of

mammals. However since the metabolism measured in this study is almost exclusively that of the "primitive red cells" these observations upon permeability do not necessarily apply to them. It would seem improbable that the oxygen consumption of cells could be increased unless the glucose actually entered the cells. A control experiment showed that no interaction, involving the consumption of oxygen,

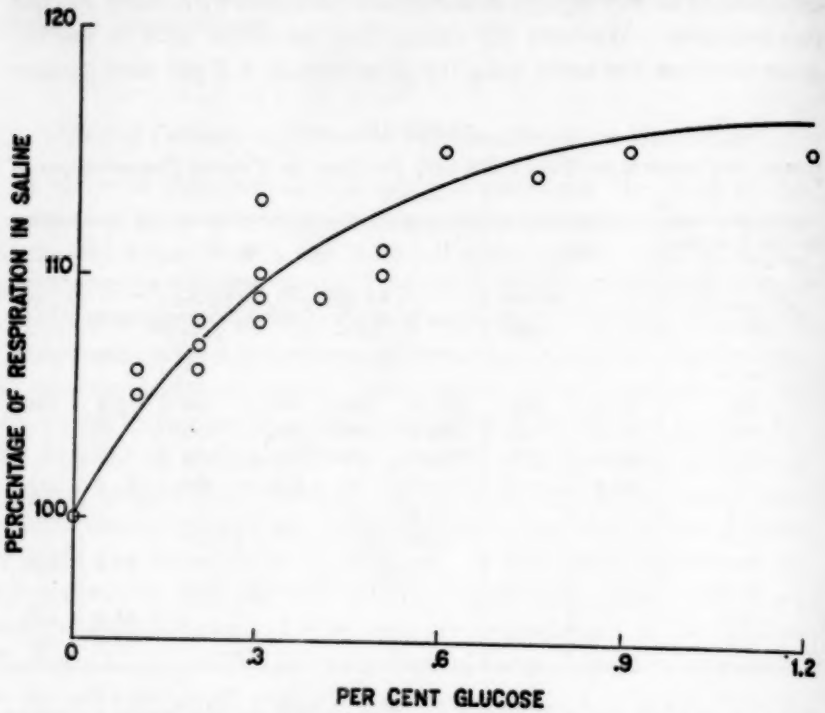

FIG. 3. Showing the influence of glucose concentration upon the oxygen consumption of primitive avian red cells.

took place when small quantities of cell-free serum were added to the glucose solutions used.

A further consideration indicating that the cells are permeable to glucose is provided by observations on the gradual decline in the oxygen consumption of the red cells as the duration of the experiment is extended. Ordinarily the period over which the observations were made was 2 hours and even in the course of that time a gradual de-

crease in the rate of consumption of oxygen could be observed. If this period were much extended the decline would become quite obvious. This decrease however was much less rapid in those solutions containing much glucose than in those with none. It would consequently seem that the oxidative processes involve the gradual depletion of some constituent of the cell which the presence of glucose in the surrounding medium could in some degree replace. This confirms the results of Grafe (17) who found that in Locke's solution containing glucose the cells respired almost as vigorously in a second experimental period of 90 minutes as they did in the first one of the same length of time.

The nature of the substances oxidized in the red cell is not known. Harrop and Barron (18) have carried out a few experiments upon the respiration of avian (goose) red cells, both in normal and anemic blood, in which they determined both the oxygen consumption and the carbon dioxide production. The resulting respiratory quotients were however too variable to give any indication of the character of the materials consumed. That these materials are, however, mainly within the cells themselves is shown by the fact that the oxygen consumption of cells suspended in an isotonic saline solution is not greatly inferior to that of cells from the same source in their own serum.

V

The Influence of Certain Amino Acids on Metabolism

Studies upon the metabolism of man and animals have shown that proteins and products of protein hydrolysis possess an especially great specific dynamic action. The following group of experiments was carried out to determine whether this stimulation of metabolism by amino acids could be reproduced on avian red blood cells *in vitro*.

In these experiments, as in those in which glucose was studied, the serum was separated from the cells by centrifugalization and replaced by solutions of various amino acids in 0.87 per cent sodium chloride. The amino acid solutions were neutralized with sodium hydroxide to pH 7.6. The buffering power of such solutions is very slight compared to that of the cells to which they were added. In each experiment the amino acid solutions added to the cells varied in concentration from

zero upwards to 50 mg. amino acid nitrogen per 100 cc. In a previous series of determinations I have found that serum of normal starving fowls contains from 14 to 18 mg. amino acid nitrogen per 100 cc. as estimated by the colorimetric method of Folin and Wu. Consequently the maximum concentration of amino acid used was approximately three times as great as in the serum of the starving fowl.

TABLE IV

Effect of Amino Acids upon the Oxygen Consumption of Red Cells

Amino acid	Millions per c.mm.	Concentration of amino acid (mg. amino *N* per cent)							
		nil.	1 mg.	2 mg.	3 mg.	5 mg.	10 mg.	25 mg.	50 mg.
		C.mm. O_2 consumed per billion red cells per hour							
Glycine	1.82	34.7 (100)				33.5 (97)	33.5 (97)	30.2 (87)	29.2 (84)
	1.39	92.8 (100)				90.9 (98)	85.3 (92)	72.0 (78)	66.1 (71)
	1.67	34.7 (100)	34.8 (100)	34.7 (100)	34.2 (99)	33.3 (96)			
Alanine	1.47	44.3 (100)	44.8 (101)	45.9 (103)	44.7 (101)	44.8 (101)	44.5 (100)		
Histidine	1.58	48.1 (100)				47.0 (98)	47.1 (98)	46.4 (97)	
l-aspartic acid	1.17	55.3 (100)				55.2 (100)	55.5 (100)		

The figures in brackets represent the oxygen consumption in percentage of that taking place in the saline medium.

The actions of four amino acids were investigated: glycine, alpha alanine, histidine and *l*-aspartic acid. The latter was studied because Burge, Wickwire, Estes, and Williams (19) had found that certain optically active amino acids, amongst them *l*-aspartic acid, exerted a stimulating effect upon the metabolism of *Paramecium*, which was not present in the optically inactive ones. Glycine and alanine on the other hand are of interest since Lusk (20) has shown them to have

especially high specific dynamic actions when tested on the metabolism of dogs.

The results are shown in Table IV.

It is clearly evident from these experiments that none of the amino acid solutions employed caused any elevation of the oxygen consumption of the fowls' red cells in any way comparable to their specific dynamic action in the intact animal. At low concentrations their effect is negligible; alanine being the only one to produce any increment and that of an order little greater than the experimental errors of the determinations. At higher concentrations than 5 mg. of amino acid nitrogen per 100 cc., glycine caused a very definite and increasing reduction in the oxygen consumption of the cells. The other three amino acids, alanine, histidine, and *l*-aspartic acid were without effect in concentrations up to 10 mg. of amino acid nitrogen per 100 cc., a concentration which it is unlikely that they ever exceed in the serum of the intact animal even at the height of digestion.

The particular component of the amino acids responsible for the specific dynamic action has long been a subject of controversy. Grafe (17) (21) has contributed to the problem from two aspects. Working originally with Warburg and employing his methods, he made a study of the action of ammonia and of a number of amines upon the oxygen consumption of the red cells of the blood of anemic geese. He observed that if 10 cc. of a neutralized saline solution containing 1/100 N NH_4Cl were added to 2 cc. of a concentrated suspension of red cells, their metabolism became approximately 60 per cent greater than those in a similar cell suspension to which saline only had been added. If, however, the cells were washed three times with this ammonium chloride solution before the oxygen consumption determinations were made the metabolism of the cells fell to approximately one-fifth of those in saline. This he accounted for by the specific absorption of ammonia by the cells, each washing contributing an additional quota to the cell contents. Consequently after three washings the cells contained considerably more ammonia than they would have absorbed from a single immersion in a solution of the same concentration. Provided the concentration of ammonia were not raised to a point at which hemolysis occurred, the inhibition of metabolism was reversible, for washing the cells from the ammonia

solutions in normal physiological saline restored the oxygen consumption to its original level. Apparently immersion in a saline solution containing 1/100 N NH_4Cl (14 mg. ammonia nitrogen per cent) raised the metabolism very considerably while the higher concentrations consequent upon repeated washings in the same solution resulted in a very considerable though reversible inhibition of the oxygen consumption.

Grafe's second contribution to this question was his study of the relative specific dynamic actions of various amino acids and ammonium chloride upon both men and animals. From the combination of his own observations with those of Lusk, Grafe comes to the conclusion that the specific dynamic action of the various amino acids is closely related to their nitrogen content. This, taken in conjuncture with the observation that, though containing no calorie content available to the body, ammonium chloride produces a pronounced stimulation of metabolism in man, makes him suggest that all these substances produce their action by reason of the amino groups which they contain. His experiments with ammonium chloride on rabbits and dogs were inconclusive.

I have carried out certain observations upon the effects of ammonium chloride at various concentrations upon the oxygen consumption of the red cells in the blood of anemic fowls. The solutions of ammonium chloride were made up and neutralized to a pH of 7.6 in exactly the same way as for the amino acids. The solutions in various concentrations were added to the concentrated cell suspensions to replace the serum removed after centrifugalization. In this way they resemble the experiment of Grafe in which he observed a very considerable stimulation of metabolism. The results are to be found in Table V.

The results of these experiments are somewhat equivocal. In all five the effect of the ammonium chloride was to produce a rise in the oxygen consumption of the cells. In two fowls, one examined twice, this stimulation was slight and attained a maximum at concentrations of between 1 and 2 mg. of nitrogen per 100 cc. In higher concentrations the metabolism of these bloods showed a progressive decline. Even at the maximum the elevation of metabolism was slight, being only approximately 5 per cent. In one fowl, also examined twice,

increase in the concentration of the ammonium chloride produced a rise in the oxygen consumption that continued as far as the maximum concentration used in experiments. The cause of the divergency in the reaction of the red cells to the ammonium chloride solutions is not evident and has not been further investigated. One difference between the blood of this animal and the others was the low oxygen

TABLE V

Effect of Ammonium Chloride upon the Oxygen Consumption of Red Cells

Fowl Number	Red blood corpuscles. Millions per c.mm.	Concentration of ammonium chloride (mg. N per cent)					
		nil.	1 mg.	2 mg.	5 mg.	10 mg.	25 mg.
		C.mm. O_2 consumed per billion red cells per hour					
2	2.36	17.8 (100)	19.0 (107)	20.1 (113)	20.5 (115)	20.1 (113)	21.1 (119)
4	1.54	37.0 (100)	38.1 (103)	38.8 (105)	36.3 (98)	35.1 (95)	34.7 (94)
5	1.91	35.4 (100)	37.2 (105)	37.2 (105)	35.6 (100)	33.2 (94)	33.0 (93)
2	1.98	15.7 (100)	17.2 (109)	17.9 (114)	18.6 (118)	18.9 (120)	19.4 (123)
5	1.74	47.4 (100)	49.0 (105)	48.6 (104)	46.3 (98)	42.3 (89)	40.7 (86)

The figures in brackets represent the oxygen consumption in percentage of that taking place in the saline medium.

consumption per billion red cells per hour, a figure only about twice that of the blood of normal fowls.

Grafe (17) in his experiment used a solution of ammonium chloride to which a molecular equivalent of sodium hydroxide had been added, so releasing free ammonia in the solution. The concentration of this ammonia was 14 mg. of ammonia nitrogen per 100 cc. Such a solution must have had an alkaline reaction at the time of its addition to the cells, though the presence of carbon dioxide in the cell suspension would ensure its rapid conversion into ammonium carbonate. In

his experiment with the red blood cells of anemic geese he observed a rise in oxygen consumption of 60 per cent. Such an elevation is far greater than any observed in the bloods of the fowls examined here. At concentrations of ammonia nitrogen comparable to his, two of the fowls' bloods showed a decrease of about 7 per cent, while the other showed an elevation of about 17 per cent.

CONCLUSIONS

1. The oxygen consumption of normal and "primitive red cells" of fowls' blood has been determined at intervals in the course of an anemia produced by the injection of phenylhydrazine. The "primitive red cells" have an oxygen consumption at least twenty to twenty-five times greater than the normal red cells.

2. Suspension of the cells derived from the blood in anemia in sodium chloride solutions of various concentrations has comparatively little effect upon the oxygen consumption of the cells.

3. The red cells from anemic blood are sensitive to variations in the reaction of the medium in which they are suspended. The maximum oxygen consumption, after addition of a saline solution containing variable amounts of acid to the blood, took place at pH 7.75. They appeared somewhat more sensitive to variations on the acid side of this reaction than on the alkaline.

4. Addition of glucose to the medium increased the oxygen consumption of the cells. Their metabolism in a physiological saline solution containing 0.6 per cent of glucose was 15 per cent higher than in one in which no glucose was present.

5. Certain amino acids in low concentrations had little effect on oxygen consumption, though at higher concentrations some of them definitely diminished it.

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FACTORS INFLUENCING THE RESPIRATION OF ERYTHROCYTES

II. Mammalian Reticulocytes

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(Accepted for publication, July 28, 1930)

Cohnstein and Zuntz (1) were the earliest investigators who clearly pointed out that certain types of blood exhibit a metabolism involving the disappearance of oxygen and reduction of the hemoglobin. This metabolism was most evident in fetal blood, which, they observed, gradually darkened in color upon standing and upon evacuation liberated less oxygen than before. They ascribed this loss of oxygen to the action of the red cells, many of which, at the stage of development of the blood which they examined, still possessed large vesicular nuclei.

Since then the metabolism of the blood has been very actively studied, notably by Warburg, by Morawitz, and by Harrop. The rather extensive literature has been well reviewed by Morawitz (2). In general, it may be summarized by stating that it is now agreed that the normal orthochromatic erythrocytes, which form the vast majority of the circulating red cells of mammals, have a negligible if any respiration (Daland and Isaacs (3)). On the other hand the polychromatic cells, which have been recently liberated from the bone marrow and which are found in large numbers in young animals and during active blood regeneration, exhibit an easily measurable consumption of oxygen and formation of carbon dioxide. The earlier nucleated cells, ordinarily present only in the bone marrow in mammals, and the nucleated circulating erythrocytes of birds and amphibia, also respire very actively.

With the exception of Warburg's studies (4), which were largely concerned with the manner in which respiration was inhibited by

various narcotics, the factors which influence this metabolism have not been extensively investigated. This paper deals with the variations in the respiration of the reticulocytes of the rabbit brought about by various alterations in the medium in which they are suspended.

Methods

The majority of the methods used in this investigation have already been described in the previous paper. The blood used was obtained from the ear vein and was defibrinated as it was collected by shaking with glass beads. Before use it was filtered through cotton wool to remove the coagulated fibrin and with it the large majority of leucocytes (Warburg (5)).

The rabbits were made anemic by intraperitoneal injections of phenylhydrazine hydrochloride (15 mg. per kilo) dissolved in normal physiological saline solution. The anemia reached its greatest severity in about 7 days. The blood used in these experiments was removed from animals at a time when there was a large proportion of reticulocytes and consequently a high rate of respiration.

The Respiration of the Blood during the Onset and Recovery Stages in Phenylhydrazine Anemia

An injection of phenylhydrazine into a rabbit is rapidly followed by the development of anemia. With suitable quantities of the substance (15 to 20 mg. per kilo) the red cell count may be caused to fall from its normal value of rather over five million to between one and two million red cells per c. mm. Regeneration, however, sets in very quickly, and by the time that the anemia has become most severe, between the sixth and eighth days, the blood may contain enormous numbers of reticulocytes. When the red cell count falls below two million per c. mm., more than 50 per cent of the cells may be reticulocytes, or more than one million per c. mm. More commonly the number lies between six and eight hundred thousand per c.mm. If the oxygen consumption of such a specimen of blood be determined it will be found to have a very much higher value than that from a normal rabbit. Harrop (6) has shown that a rough proportionality exists between the number of reticulocytes in, and the oxygen consumption of, various pathological human bloods. Similarly Derra (7) studied the oxygen consumptions of blood during the remissions resulting from the administration of liver extract in patients suffering from pernicious anemia. He found a similar relationship, though not so close as that

observed by Harrop, between the oxygen consumption and the reticulocyte content of the blood at various times in the remissions.

In the following experiment two rabbits were made anemic by injection with phenylhydrazine. Red cell counts, reticulocyte counts and oxygen consumption determinations were made every second day until the main wave of liberation of reticulocytes had subsided. The observations are to be found in Table I and Fig. 1.

TABLE I

The Oxygen Consumption of Reticulocytes at Various Phases in the Anemia Induced by the Injection of Phenylhydrazine

Rabbit No.	Day	Red cells in mills. per c.mm.	Retics. per cent	Retics. in mills. per c.mm.	C.mm. O_2 consumed	
					Per cc. blood per hour	Per billion retics. per hour
28	before inject.	5.49	1.5	0.083	5.1	63.0
	1st after inject.	6.01	3.2	0.192	11.1	57.8
	3rd " "	2.85	9.3	0.265	17.0	64.2
	5th " "	2.40	27.0	0.649	41.9	64.7
	7th " "	2.44	33.0	0.805	52.9	65.5
	9th " "	3.80	17.2	0.654	35.5	54.3
	11th " "	4.07	10.2	0.423	25.4	59.9
	13th " "	4.90	9.6	0.470	26.0	55.3
40	before inject.	4.97	2.0	0.099	5.3	53.6
	1st after inject.	4.96	3.0	0.149	8.9	59.7
	3rd " "	3.32	9.4	0.313	14.9	47.6
	5th " "	2.64	23.9	0.631	40.0	63.2
	7th " "	3.15	21.0	0.630	35.2	55.9
	9th " "	3.67	13.1	0.479	25.3	52.8
	11th " "	3.19	9.1	0.290	15.2	52.4
	13th " "	3.61	5.3	0.192	10.9	56.7

From Table I it can be seen that the actual number of reticulocytes per c.mm. of blood increased, in one animal to more than nine, in the other to more than six, times their initial figures, and that the oxygen consumption of the blood underwent a concurrent change. But in spite of the very large alteration in both, the oxygen consumption for every billion (thousand million) reticulocytes was, within reasonably close limits, the same throughout the course of the experiment.

There are at least two possible explanations for this rather strikingly uniform relationship between oxygen consumption and reticulocyte concentration. In the first place it is possible that the reticulum in the cell either promotes oxidation or is itself oxidized at the same rate at all stages in its gradual disappearance. When taken in conjunction with the cytological appearances of the reticulocytes with their very variable content of basophil filaments, such an assumption seems

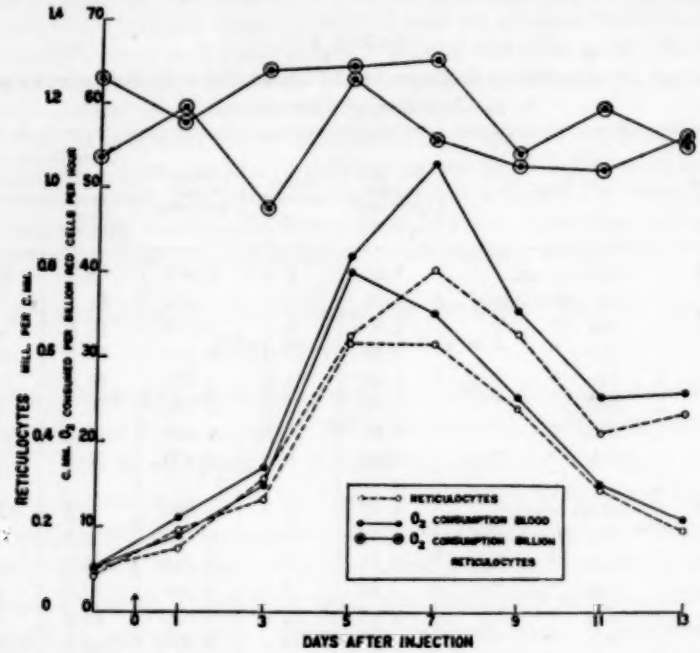

FIG. 1. Showing the oxygen consumption of reticulocytes related to their concentration in the blood at various stages of an anemia produced by phenylhydrazine.

hardly probable. A second and seemingly more likely explanation is that the reticulocyte stage of development is of comparatively short duration in comparison with the whole duration of the wave of reticulocyte production evoked by the phenylhydrazine. Under these circumstances the oxygen consumption curve for the blood would represent, at any one particular time, the summation of the oxygen consumption of reticulocytes at all stages in their life history. In this

event, though the total number of reticulocytes in the circulation might change greatly, the proportions of the various categories of cells, young and old, might not be very different at different times.

An accurate estimation of the duration of the reticulocyte stage of development would determine this point. Subject to some assumptions which will be discussed below, this period can be indirectly estimated from a consideration of the regeneration curves for the blood of rabbits recovering from anemia. In the course of this recovery the red cell count of the animal rises from the day of most severe anemia until regeneration is complete. If daily total red cell counts and reticulocyte percentage counts be made, the actual number of reticulocytes per c. mm. of blood can be calculated for any particular day. Should, on an average, each reticulocyte retain its reticulum for only 1 day before developing into an ordinary orthochromatic erythrocyte, it will, taken in the aggregate, be only counted once as a reticulocyte in the daily blood examinations made. Consequently, if the life of the reticulum once the cell has reached the circulating blood be only 1 day, the total daily calculated numbers of reticulocytes added together for the whole regeneration period should be equal to the rise in the red cell count during the same period. If the aggregate number of the reticulocytes so counted be greater than the number of new cells produced, as judged by the rise in the total red cell count, the average duration of the reticulum in the cells must be longer than 1 day, since they must have been counted more than once.

Such a calculation obviously makes several important assumptions. First, it presupposes that all the red cells added to the circulation in the period of regeneration are liberated from the bone marrow as reticulocytes and not as the normal adult orthochromatic variety of red cell. It is impossible at the present time to be certain whether this is so or not, since there are no satisfactory ways of recognizing new cells other than those of vital staining. If considerable numbers of non-reticulated red cells are liberated the calculation just outlined will estimate the duration of the reticular stage of development at too brief a period. It further disregards any alteration in blood volume and the normal or abnormal destruction of red cells, either adult or reticulated, during the regeneration period. How far these might affect the result it is clearly impossible to estimate.

With all these assumptions, only a very roughly approximate estimate is possible, yet the calculation seems to be worth the making. Table II contains data taken from a group of animals used in another experiment.

The agreement between the results on these animals is not good. Nevertheless, it would seem that the duration of the persistance of the reticulum in the red cell after it has left the bone marrow is probably not much greater than 2 days, and that in severe grades of anemia it may be even less. Such a period is much briefer than the duration of the entire reticulocyte wave following an injection of phenylhydrazine. Further it would support the view already suggested that the uniformity of the oxygen consumption per billion reticulocytes throughout the period of the anemia results from this consumption being largely

TABLE II

Rabbit No.	Red cell counts			Tota lnumber of retics. counted	Tota lretics.
	Lowest	On recovery	Rise		Red blood corpuscle rise
104	3.77	5.72	1.95	4.13	2.12
105	3.82	5.70	1.88	4.10	2.18
106	2.20	5.11	2.91	3.19	1.10
103	1.14	5.47	4.33	6.22	1.44

or even exclusively due to the metabolism of those cells which had first appeared in the blood stream in the previous 36 to 48 hours.

Estimations of the duration of the reticulocyte stage of development have been made by Pepper (8) and by Heath and Daland (9), both largely from observations upon the rate of disappearance of reticulocytes from regenerating blood incubated at 37°C. Pepper states that in no specimen of blood left in the incubator were fully formed reticulations found after 48 hours, but in one instance a few remnants of reticular substance were found after 66 hours incubation. Heath and Daland used a variety of methods, all of which gave consonant results, and showed that reticulocytes of the rabbit persist for a period varying from 48 to 72 hours, in incubated blood, after introduction into the pleural cavity of the rabbit and probably also after transfusion into a normal rabbit. A few persisted longer, but the large majority had

disappeared in that time. These results are in fairly good agreement with those calculated from the regeneration curves.

The Influence of Hydrogen Ion Activity upon the Respiration of Reticulocytes

The hydrogen ion activity in the interior of the mammalian red cell has been found to depend upon the reaction of the medium in which it is suspended. The divergencies between the reactions inside and outside the cell have been investigated notably by Warburg (10), by Van Slyke and his collaborators (11), and by Henderson (12). At reactions between pH 6.5 and pH 6.9 the hydrogen ion activity of the interior of the cell and of the surrounding medium are approximately the same, but, as the alkalinity of the surrounding medium increases, that of the cell increases less rapidly so that they become relatively acid. Through the region of physiological neutrality the difference between the two is not very considerable (at pH 7.4 there is a difference of about 0.1 pH), but the difference increases more than proportionately rapidly until at a serum pH of 8.0 the cell interior is at a reaction of about pH 7.75. It is evident that the contents of the mammalian red cell are capable of considerable variations in their reaction, and that these variations can be controlled by alterations in the reactions of the surrounding medium. The purpose of the present experiments was to determine how far the oxidation processes associated with the reticular substance were affected by changing the hydrogen ion activity of the medium in which the cells were suspended.

The reaction of the cells was varied by the addition of small quantities of sodium hydroxide or of hydrochloric acid to defibrinated blood. Two cc. of normal physiological sodium chloride solution containing the necessary acid or alkali were added slowly and with frequent shaking to 4 cc. of blood from an anemic rabbit. After mixing, 3 cc. of the blood saline mixture were placed in the flask of the Barcroft-Warburg apparatus and their oxygen consumption determined. Directly this had been ascertained the contents of the flask were rapidly transferred to centrifuge tubes and the cells and suspension medium separated. The reaction of the medium was determined with the hydrogen electrode. I am indebted to Dr. Arda A. Green for making most of the determinations.

TABLE III

The Influence of Hydrogen Ion Activity upon the Oxygen Consumption of Reticulocytes

Concentration of acid or alkali added	C.mm. O_2 per bill. retics. hourly	Per cent of value in serum saline	pH	C.mm. O_2 per bill. retics. hourly	Per cent of value in serum saline	pH	C.mm. O_2 per bill. retics. hourly	Per cent of value in serum saline	pH
NaOH 1/50 N.....	30.6	89					54.4	92	8.31
1/100 N.....	30.6	89	8.06				54.8	93	8.16
1/200 N.....	32.0	93	8.02				56.3	96	8.04
Neut. Sal.....	34.5	100	7.96	48.4	100	8.10	58.8	100	8.02
HCl 1/200 N.....	34.0	99	7.86						
1/100 N.....	34.1	99	7.81	46.8	97	7.89			
1/50 N.....	31.9	92	7.68	45.7	95	7.70	55.6	95	7.65
1/33 N.....				44.5	92	7.55			
1/25 N.....				39.4	82	7.34			
1/20 N.....				36.8	77	7.06	48.5	83	7.22

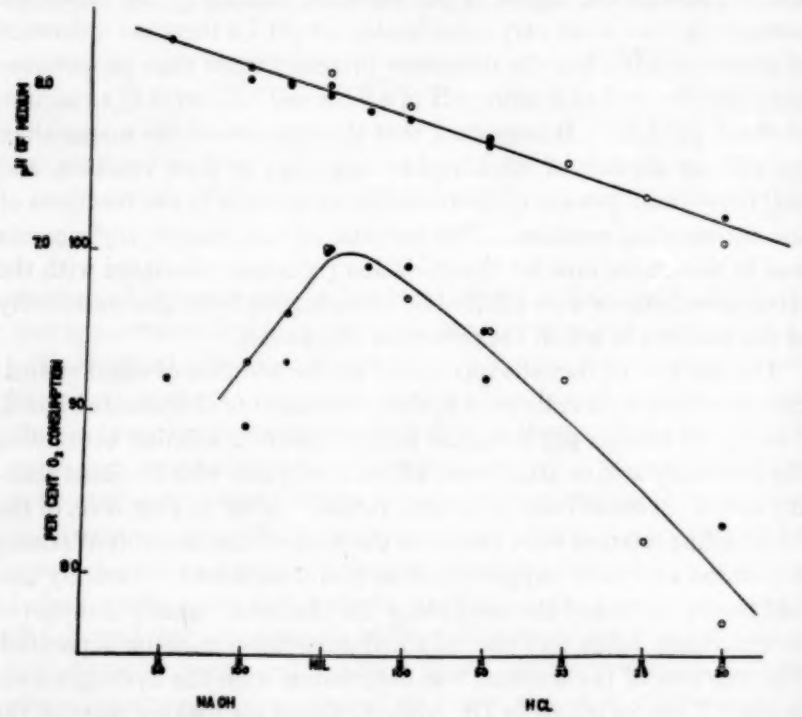

FIG. 2. Showing the influence of the addition of HCl and NaOH to blood upon its reaction and upon the respiration of the reticulocytes present in it.

The results of the experiments will be found in Table III and in Fig. 2. It will be seen that variations in the reactions of the medium have considerable influence upon the reaction of the cells. The maximal oxygen consumption occurred about pH 8.0, which would correspond to a reaction in the interior of the cells about pH 7.75. The metabolism at pH 7.4, or that normally to be found in the blood, would be about 85 per cent of this maximal value.

The Influence of Variations in Tonicity upon the Respiration of Reticulocytes

Determinations of the oxygen consumption of reticulocytes were made in solutions of sodium chloride of various concentrations ranging from 0.11 molar (0.6 per cent) to 0.28 molar (1.5 per cent). These solutions were added to the cells after they had been separated from their serum by centrifugation.

The results are given in the following table:

Red cells in mills. per c.mm.	NaCl: 0.6 per cent	0.9 per cent	1.2 per cent	1.5 per cent
	C.mm. O_2 consumed per billion reticulocytes per hour			
2.75 (14.2)*	32.5	39.4	34.9	31.2
2.37 (23.4)	38.5	44.2	39.6	37.8
1.96 (39.0)	38.9	42.9	42.5	37.8

* Reticulocyte percentages are given in brackets after the red cell counts.

It can be seen that in all three experiments the maximal respiration took place in a sodium chloride solution which was closely isotonic with normal blood. The figures, however, suggest that slight degrees of hypertonicity are less injurious to the cell respiration than the reverse condition of hypotonicity. The cells in 0.6 per cent saline have a metabolism 13 per cent below that in 0.9 per cent saline, while those in 1.2 per cent saline are only depressed to the extent of 7 per cent.

The Influence of Glucose, Glycine, and Alanine on the Respiration of Reticulocytes

In order to determine whether the presence of certain readily oxidizable substances in the surrounding medium resulted in any accelera-

tion of the rate of respiration of the reticulocytes, they were separated from their serum and added to saline solutions containing variable concentrations of these substances. Glucose was used in concentrations varying from zero to 400 mg. per hundred cc. Glycine and alanine were used in solutions containing from zero to 28 mg. of amino acid nitrogen per hundred cc. These amino acid solutions had no perceptible effect upon the rate of respiration, while the glucose solutions progressively depressed it, the respiration in saline containing 400 mg. per cent of glucose being on the average only 84 per cent of that in saline. Whether the glucose actually entered the reticulocytes is problematical. Kosawa (13), and Ege (14) found that the red cells of the rabbit are impermeable to glucose, but this may or may not be true of the reticulocytes.

Since reticulocytes have approximately the same rate of respiration in normal physiological saline solution as they have in their own serum, it is probable that the substances oxidized are present in the cells as a residue from an earlier stage of development, and are not gaining access to them continuously from the exterior. The disappearance of the reticulum during the period in which respiration takes place suggests that it is part if not the entire material consumed. The actual quantity of this substance oxidized may be approximately calculated. Since the reticulocyte stage of development is about 50 hours, and the average oxygen consumption about 60 cm. per billion reticulocytes per hour, the total quantity of oxygen used by every billion reticulocytes will be about 3 cc.

Unfortunately very little is known of the chemical nature of the reticular substance. Gawrilow (15), from a study of its reaction with vital stains, believes that it is partly protein, partly lipoid. Neither is there any consistent data upon the respiratory quotient of reticulocytes from which it might be possible to draw any conclusions as to its chemical constitution. Douglas (16) found an average respiratory quotient of 1.02 on three samples of citrated anemic rabbit's blood. This is much too high a value to be accounted for by the oxidation of either lipoid or protein, though there is no reason to believe that other substances may not be oxidized in addition to the vitally staining reticulum. Harrop and Barron (17) (1928) found values from which they suggested that the respiratory quotient was between 0.75 and

0.80, and such a value would of course be in closer agreement with Gawrilow's views.

Should the material consumed in the reticulocytes be a fatty acid, 3 cc. of oxygen would account for the oxidation of about 1.5 mg.; were it carbohydrate or protein, the weight would be greater. The weight of the dry material of one billion reticulocytes is about 40 mg., of which about 35 mg. is hemoglobin, so that the respiration of the reticulocyte must result in the destruction of a large proportion of its contents apart from the hemoglobin. This proportion is all the greater since more than 1 mg. of the residual 5 mg. is inorganic matter.

DISCUSSION

At the conclusion of this paper it is possible to compare the respiration of the mammalian reticulocytes and that of the normal nucleated and primitive red cells of the bird. Such a comparison is necessarily rough, but it may afford some indication of the solution of the problem which was raised at the commencement of the first paper of this series: the relative importance of the nucleus as the seat of cell oxidation. On the one hand, in the rabbit reticulocytes, we are concerned with a corpuscle entirely devoid of a nucleus; on the other, in the primitive avian erythroblast, a nucleated cell possessing an intensity of respiration not greatly inferior to that of a parenchymatous cell such as the liver cell.

Since all the determinations were made at the same temperature (37.5°C.) no allowance need be made for the temperature coefficients of the respiratory processes. There are, however, considerable differences in the sizes of the various cells whose respiration is to be compared. Consequently, in order to render the comparisons more satisfactory, the respirations should be considered not on the basis of cell number but rather upon one of cell volume. This is evident since the volume of the primitive red cell of the fowl is about twice as great as that of the rabbit's reticulocyte.

Hematocrit observations make it possible to determine the volume occupied by the various types of red cell. The volume of one billion red cells in the blood of anemic fowl is approximately 140 c.mm., in the blood of a normal fowl 100 c.mm., and of the reticulocytes of the rabbit 85 c.mm. In the blood of the anemic fowl about 50 per cent

ot the cells were the normal variety of adult erythrocyte, so that the volume occupied by one billion of the more primitive cells would be about 180 c.mm. The following table shows the relationship between the respiration of these types of cells, together with their volumes, and allows a comparison to be made of their respiration on the basis of an arbitrary unit volume of 100 c.mm.

Type of cell	Volume occupied by one billion cells	Oxygen consumption per billion cells	Oxygen consumption per 100 c.mm. cells
	c.mm.	c.mm.	c.mm.
Fowl: Prim. Red Cell	180	250	140
Fowl: Norm. Red Cell	100	12	12
Rabbit: Retics	85	60	70

It is evident from this table that if the metabolism of the reticulocyte gives any indication of the intensity of oxidation taking place in the cytoplasm of a cell such as the primitive red cell of the fowl, that the oxidative processes of the cell are far from taking place exclusively in relation with the nucleus. The reticulum appears to be associated closely with the cell oxidation either as the material consumed or as the mechanism by means of which the process is effected. The possibility that the reticulum is in reality a nuclear remnant is now generally discredited. Both the primitive red cells of fowls and many of the normoblasts of mammals possess both a nucleus and a reticulum, and both are often present simultaneously in the cells as distinct structures. Further, their staining properties are dissimilar. It would seem probable, therefore, that in the primitive avian red cell oxidation processes are taking place both in the cytoplasm and in the nucleus and that the partition between the two may not be far from equal. Further, it is evident that though the definitive red corpuscles of the fowl are nucleated cells the respiration is small in comparison with the non-nucleated reticulocyte of the mammal with its residual cytoplasmic reticulum.

SUMMARY

1. The respiration of the reticulocytes of the rabbit has been measured during the period of an anemia produced by phenylhydrazine. Though the respiration increased greatly during the phase

of regeneration, the oxygen consumption per billion reticulocytes throughout the period remained approximately the same.

2. The respiration of the reticulocytes was affected by changes in the reaction of the medium in which they were suspended, and was at its maximum about a pH of 8, with a probable intracorpuscular pH of about 7.75.

3. Variations in the tonicity of the suspending medium did not produce any great change in the respiration of the reticulocytes.

4. The presence of glycine, alanine, and glucose in the suspending medium resulted in no acceleration in the respiration of the cells. At higher concentrations glucose tended to depress the respiration. The material oxidized appears to be mainly or entirely contained in the corpuscles at the time they are liberated from the marrow.

5. A comparison is made of the respiration of the reticulated nucleated red cells present in the blood of anemic fowls and the non-nucleated reticulated red cells of rabbits. On the basis of equal volumes of cells, the respiration of the former is about twice that of the latter, while this in turn is about six times as great as the nucleated but non-reticulated normal red cells of the fowl.

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THE EFFECT OF SALTS ON THE IONISATION OF GELATIN

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(Accepted for publication, August 4, 1930)

The internal salt, or "zwitter ion" theory of the structure of amino acids, has received increasing support of recent years. In the case of proteins, however, the data are less certain. According to the classical theory, gelatin is neutral at the isoelectric point by virtue of being either completely non-ionised or else containing a minimum number of dissociating groups, while the zwitter ion structure explains its behavior at this point on the basis of its possessing equal and maximal numbers of positive and negative charges. Such a molecule, with charges distributed over the surface, should be as capable of combining with a neutral salt as with an acid or base, and any such protein-salt compounds, since they involve at least one strong electrolyte, would be expected to be dissociated. Thus:

$$\overset{-+}{\text{protein}} + \overset{+-}{\text{NaCl}} = \overset{+}{\text{Na}}\ \overset{-+}{\text{protein}} + \overset{-}{\text{Cl}}$$

$$\overset{-+}{\text{protein}} + \overset{+-}{\text{NaCl}} = \overset{-+}{\text{protein}}\ \overset{-}{\text{Cl}} + \overset{+}{\text{Na}}$$

On the other hand, it is extremely difficult to postulate a mechanism whereby the neutral molecule of the classical theory could combine with neutral salt to produce a highly ionised compound, and even in the case of a molecule which bears a very small residual number of charges, any such combination could only be of a very slight order. The existence in considerable amounts of such compounds with neutral salts would therefore be indirect evidence for the zwitter ion structure of protein molecules.

The purpose of this paper is to draw attention to the fact that much of the data already published, particularly by Loeb and his co-workers, contain implicit evidence that the addition of neutral salts to

gelatin solutions increases the ionisation of the gelatin, *i.e.*, that the gelatin therefore combines with neutral salts to produce highly dissociated complexes.

Now if a gelatin solution be contained within a membrane permeable to electrolytes which is immersed in acid, it follows from the Donnan membrane relationship that the concentration of diffusible ions within the membrane is different from that outside it.

In the case of gelatin-HCl solution, on the acid side of pH 4.7, the H-ion concentration is less within the membrane than without, and the Cl-ion concentration is hence greater within the membrane, since

$$[H]_{in}\,[Cl]_{in} = [H]_{out}\,[Cl]_{out}$$

There is therefore an excess of the Cl-ions within the membrane, that is an excess of negative ions, and some of the gelatin must therefore be positively ionised to maintain electrostatic balance. If the H-ion activities are measured, the concentrations of all ions, including that of ionised gelatin, can be calculated. In the presence of known concentrations of salts the conditions are the same, and if the gelatin solution be at the same pH as when the salts are absent, then the comparison of the amounts of gelatin ionised in the two cases would give the effect of the addition of salt on the ionisation of gelatin.

Data on this point are readily available in the literature. The measurements at pH 3.8 are taken from Loeb (1) and Loeb and Kunitz (2). These determinations refer to the solutions at the beginning of the experiment, and in each case it has been necessary to assume that the pH of the outer solution has remained unchanged throughout. This is unavoidable since, not final pH measurements, but only membrane potentials, are given. Now the establishment of a membrane potential is merely the necessary consequence of a difference in concentration of H^+ or Cl^- ions, from whatever cause, since:

$$\text{E.M.F.} = 59(pH_y - pH_x)$$

The agreement between the membrane potential observed and that calculated from the pH difference does not therefore possess any special significance, since it does not of itself confirm the Donnan relationship, but only checks the accuracy of the measurements. Even

if measurements of final pH within and without had been available, however, the correction to be made for the probable slight change in the pH of the outer solution would have been unimportant, since it is merely a difference of pH that is involved. Furthermore the volume of the outer solution was much greater than that of the inner and the pH change was therefore probably slight.

TABLE I

Gelatin Chloride and Sodium Chloride. (1) and (2). 1 per cent Gelatin = 9 Milliequivalents Per Litre

In presence of sodium chloride; outer pH = 3.8								In absence of sodium chloride; $\gamma_H = 0.99$				
Concentration of salt	− logα molality	γ_H	Membrane potential millivolts	C_{H^+} internal milli-equivalents per litre	C_{Cl^-} internal	C_{Na^+} internal	$C_{\text{gelatin ions}}^{+}$	Membrane potential millivolts	C_{H^+} external	C_{Cl^-} internal	$C_{\text{gelatin ions}}^{+}$	Ratio
M:8	0.9	0.84	0	0.189	125.0	125.0	0	32.5	0.581	2.11	1.95	
M:16	1.2	0.87	0.2	0.181	63.20	62.00	1.02	32.5	0.577	2.10	1.94	0.52
M:32	1.5	0.90	1.0	0.172	32.16	30.53	1.46	32.5	0.567	2.05	1.90	0.77
M:50	1.7	0.90	2.0	0.164	21.62	18.66	2.80	33.0	0.542	1.97	1.82	1.54
M:100	2.0	0.92	3.5	0.152	11.54	8.81	2.58	33.5	0.518	1.90	1.76	1.47
M:200	2.3	0.95	6.5	0.131	6.57	3.93	2.51	33.5	0.462	1.70	1.57	1.60
M:400	2.6	0.97	11.0	0.107	4.11	1.62	2.38	33.0	0.384	1.40	1.30	1.83
M:500	2.7	0.97	13.0	0.099	3.61	1.20	2.31	33.0	0.350	1.26	1.16	1.99
M:1000	3.0	0.98	18.0	0.082	2.34	0.497	1.76	33.0	0.280	0.99	0.914	1.93
M:2000	3.3	0.98	24.0	0.063	1.67	0.197	1.41	33.0	0.231	0.846	0.783	1.80
M:4000	3.6	0.99	29.0	0.050	1.30	0.079	1.18	32.5	0.182	0.656	0.606	1.93
M:8000	3.9	0.99	31.0	0.048	0.955	0.037	0.870	32.5	0.172	0.621	0.573	1.52
M:16000	4.2	1.0	33.0	0.044	0.811	0.017	0.749	32.0	0.162	0.596	0.552	1.36

The first section of Table I gives the salt concentration and observed membrane potentials, the outer solution being at pH 3.8. From the membrane potentials were calculated the H-ion activities within the membrane, and from these the H-ion concentrations by use of Lewis and Randall's figures (3) for the activity coefficients of the H-ions in solutions of various strengths (Column 3), neglecting, of course, the unknown contribution of the gelatin to the ionic strength of the solution. The concentrations of the other ions, and therefore of the

gelatin which must be ionised to produce electrostatic equilibrium, follow. The second section gives the figures for a gelatin solution at the same pH as in the first section, but in equilibrium with HCl only. The last column shows the value of the ratio:

$$\frac{\text{Gelatin ionised in presence of NaCl.}}{\text{Gelatin ionised in absence of NaCl at same pH.}}$$

This ratio is plotted in the curve, the abscissae being the negative logarithms of the molality of the salt solution; this scale is simpler than plotting direct reciprocals of molality and corresponds closely to the pH scale.

The figures show clearly that apart from the depression of ionisation produced by very concentrated salt solutions in acid, the addition of salt to the gelatin-HCl or gelatin-NaOH systems increases the ionisation of the gelatin. The variation of the ionisation ratio in the pH range 3.8 to 4.3 reaches a maximum corresponding to about M/1000 NaCl solution. The curve is, however, the resultant of two effects, (a) of decreasing salt concentration from M/8 to M/16000 and (b) of increasing pH from 3.8 to 4.3, and at constant pH would be slightly flatter.

The effect of salts in depressing the osmotic pressure, viscosity and swelling of gelatin, as shown by Loeb, is to be considered quite apart from this increase in ionisation of the gelatin, which is most simply explained on the formation of complex salts.

The observations of Pauli (4) and (5) on electrolyte-free proteins, show from conductivity and ionic mobility measurements that proteins combine with zinc chloride to produce complex zinc-protein ions, positively charged, and also, under certain conditions, to give complex chlorine-protein ions. Northrop and Kunitz (6) also find that gelatin combines readily with calcium, copper and other salts to produce positively charged particles, and that the combination even close to the isoelectric point, becomes, in high salt concentrations, stoichiometrically equivalent to the combination with hydrogen.

In this connection it is possible to show that gelatin possesses an effective combining power toward metal ions of from 2.5 to 3.6, for the formation of complex ions under the conditions of the experiment. In Table II are given the concentrations, in millimols per 1000 gm.,

for Donnan systems of gelatin with calcium and copper chlorides. Column 1 gives the external concentration of the salt, as given by Northrop and Kunitz, and the second and third columns give the in-

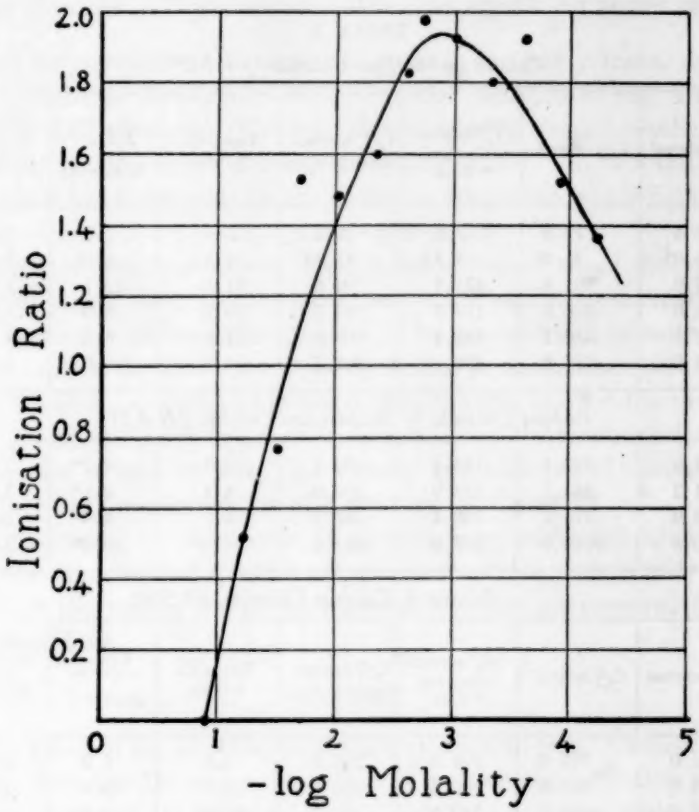

FIG. 1. Ionisation of gelatin produced by adding salt solution, from data of Loeb and Kunitz. The ratio of the amount of gelatin ionized in presence of NaCl to the amount ionized without NaCl but at the same pH is plotted against concentration of NaCl solution.

ternal concentrations of chloride and metal ions calculated from the membrane potential. Just as in Table I, there should be an excess of negative ions, and this is given by a (Column 6). Now the amounts of copper found, $[Cu]_2$, are in excess of the theoretical by a consider-

able amount, $[Cu]_3$, which, since it is not subject to the Donnan relationship, cannot be present in the normal ionic form. It can only be present in association with the gelatin that has to be positively

TABLE II

*Gelatin and Copper Chloride, pH 5.0***

C_{Cu} external	C_{Cl^-} internal	$C_{Cu^{++}}$ internal calculated $= [Cu]_1$	$C_{Cu^{++}}$ internal found $= [Cu]_2$	Excess Cu^{++} $= [Cu]_3$	$\frac{C_{Cl}}{2} - [Cu]_1 = a$	Effective combining power $= \frac{[Cu]_3}{a} \times 2$
8.8	19.6	7.1	10.7	3.6	2.7	2.67
8.05	21.97	4.32	13.15	8.83	6.66	2.65
187.9	391.5	173.1	204.6	31.5	22.7	2.77
178.0	360.0	174.0	184.5	10.5	6.0	3.50
192.5	389.3	188.4	199.5	11.1	6.3	3.52
485.0	976.0	479.0	495.5	16.5	9.0	3.66
Copper Chloride + Deaminized Gelatin, pH 4.1†						
191.8	387.1	188.4	194.1	5.7	4.7*	2.46
291.2	584.7	287.9	294.0	6.1	4.1*	2.97
383.9	771.4	380.4	386.5	6.1	4.9*	2.49
500.0	1014.0	487.0	515.0	28.0	19.6*	2.86
Gelatin + Calcium Chloride, pH 5.0‡						
C_{Ca} external	C_{Cl^-} internal	$C_{Ca^{++}}$ internal calculated $= [Ca]_1$	$C_{Ca^{++}}$ internal found $= [Ca]_2$	Excess Ca^{++} $= [Ca]_3$	$\frac{C_{Cl}}{2} - [Ca]_1 = a$	Effective combining power $\frac{[Ca]_3}{a} \times 2$
295.0	594.0	291.0	299.0	8.0	6.0	2.67
494.0	992.0	490.0	501.0	11.0	6.0	3.67
744.5	1495.0	739.0	751.0	12.0	8.5	2.82
984.0	1976.0	976.6	994.5	17.9	11.4	3.16

* Values corrected for concentration of H^+ ion.
** See Table I, Northrop and Kunitz (6).
† See Table II (6).
‡ See Table VII (6).

ionised to neutralise the excess of negative ions. In the first case, the excess of divalent negative ions is 2.7 millimols, which must therefore be neutralised by 2.7 millimols divalent positive ions. The excess of

copper over the theoretical is 3.6 millimols, and these must therefore be absorbed into the formation of 2.7 millimols divalent positive Cu-gelatin ions, so that the gelatin must be acting as though $\frac{3.6}{2.7} \times$ 2-valent. This effective valency or combining power of the gelatin for complex ion formation is given in the last column, and is seen to increase slowly with increasing concentration of salt, from a value of 2.67 upwards. Calcium chloride yields similar results; deaminised gelatin shows a lower combining power. In this last case correction has to be made for the H-ion concentration; at pH 5.0 this is negligible, at pH 4.1 it reduces the value of a by 0.4.

As in the previous tables, the figures represent small differences between large values, and the agreement is therefore very satisfactory. The formation of ionisable compounds by the addition of salts goes far to explain the solubility of the globulins in neutral salt solutions, since the idea, so often put forward, that the ions are soluble and the non-ionised protein is not, now receives a definite mechanism for the production of ions by salt. As Vickery and Osborne (7) point out, this fact has been somewhat of a mystery until now.

Furthermore, the formation of complex ions, while difficult to explain on the classical theories of protein structure, falls readily into line with the zwitter ion conception and is in fact an expected corollary therefrom.

SUMMARY

The effect of the addition of sodium chloride to gelatin solutions is shown from the Donnan relationship to increase the ionisation of the gelatin, the increase produced in acid solutions reaching a maximum at about 1/1000 molar salt concentration. This effect is attributed to the formation of complex ions.

From the similar action of calcium and copper chlorides the effective combining power of gelatin for complex positive ion formation is deduced. The bearing of complex ion formation on the zwitter-ionic structure and solubility phenomena of proteins is pointed out.

The author's thanks are due to Dr. M. Kunitz for directing his attention to some of the published measurements, to H. M. Winegar-

den, and particularly to Dr. H. Borsook for his helpful advice and criticism.

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THE KINETICS OF THE BACTERIUM-BACTERIOPHAGE REACTION

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(Accepted for publication, July 15, 1930)

The clearing of broth cultures of bacteria by an agent which produces more of itself during the period of contact with susceptible organisms constitutes a striking phenomenon, a process obviously of great interest to bacteriologists and one which accordingly has been the subject of extensive study. However, attempts to analyze the mechanism of phage action upon bacteria have not resulted in general concordant interpretation of the basic experimental facts, and indeed many of the major facts themselves are wanting or are in dispute. This is due in part to the inaccuracies involved in determining phage quantitatively and to the difficulty of estimating bacteria in the presence of phage. One of us has described experimental procedures for determining phage (1) and for estimating bacteria in phage-containing suspensions (2), both being sufficiently convenient and accurate for use in a quantitative analysis of the phage-bacterium system. The present paper deals with such an analysis in the particular case of an antistaphylococcus bacteriophage acting upon a single strain of *S. aureus*.

General Description of the Reaction between Antistaphylococcus Bacteriophage and S. aureus

Before going on to the reaction itself the terms and abbreviations used throughout should be defined.

B: Bacterium, a 16 hour agar culture of a single phage-susceptible strain of *S. aureus* suspended in saline.

[*B*]: Total number of bacteria/ml.

Bo: Initial value of [*B*].

Be: [*B*] at maximal stationary phase of *B* growth.

P: A polyvirulent highly active antistaphylococcus phage.
P.U.: Phage unit.
[*P*] or [P.U.]: Phage units/ml.
Po: Initial value of [*P*].
Broth: Veal infusion, 1 per cent peptone, 0.5 per cent NaCl, pH 7.6.
Standard phage: Antistaphylococcus bacteriophage prepared by suspending 2×10^7 staphylococci per ml. in 1 per cent phage. The mixture is rocked at 36°C. until lysis is complete. The lysate is stored at 5°C. without filtering.

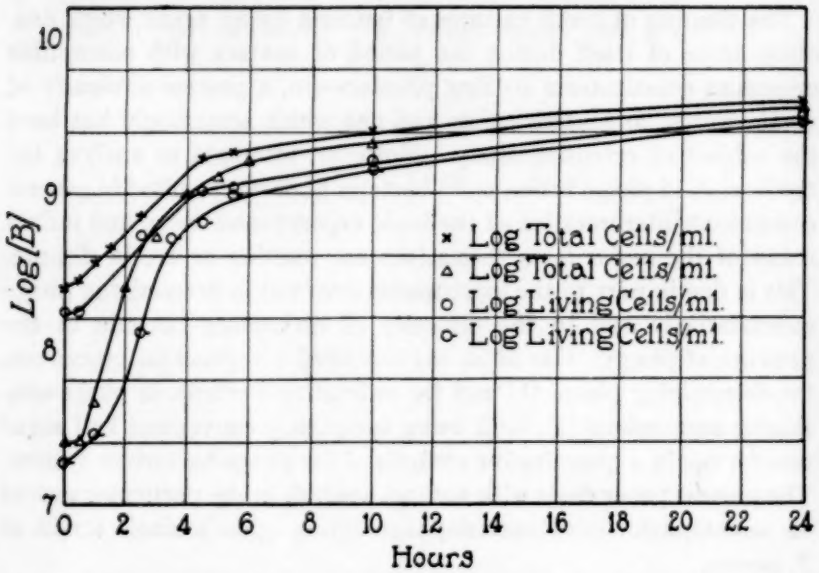

FIG. 1. Normal growth curves of *S. aureus* in broth at 36°C. Plots of log [*B*] against *t* for two different values of *Bo*. Total cells estimated by turbidity method, living cells by plate count.

For each experiment the following data were obtained at various time intervals: Total *P*/ml.; extracellular *P*/ml.; intracellular *P*/ml. (by difference); *B*/ml., in the *P*-containing suspension and also in the broth alone without *P*. Essential conditions prevailing unless otherwise indicated were: Temperature 36°C. and constant agitation obtained by placing the test series in a mechanical shaker mounted in a water bath. The methods employed in making the various deter-

minations are described at the end of the paper. The complete data furnish the basis upon which the following description of the *B-P* reaction rests.

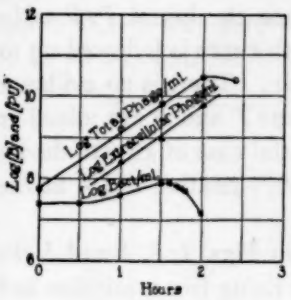

FIG. 2

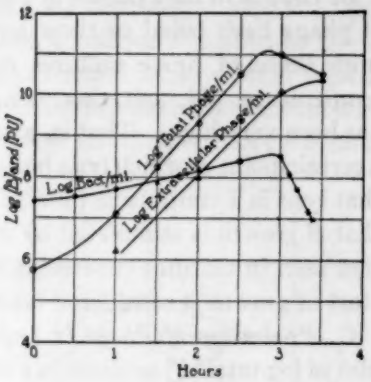

FIG. 3

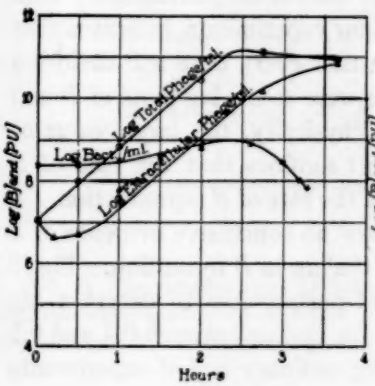

FIG. 4

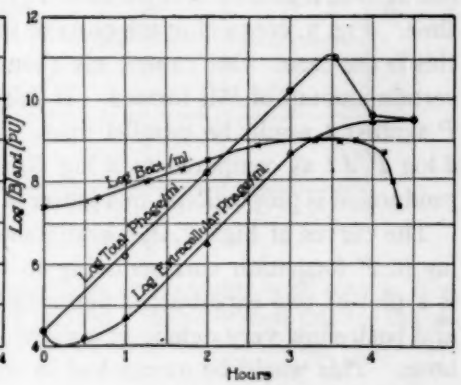

FIG. 5

FIGS. 2, 3, 4 and 5. Plots of typical kinetic experiments. The curves of log total [*P*], log extracellular [*P*] and log [*B*] against *t* for different values of *Po* and *Bo* at 36°C. are shown.

A. Normal Growth.—Normal growth of the test organism in broth at 36°C. is shown in the plot of log [*B*] against time (Figs. 1 and 11). The usual features of a growth curve are observed here, namely, a period of lag (average about 0.5 hour), a brief phase of positive

acceleration, a relatively long period of logarithmic growth, a phase of negative growth acceleration and a prolonged maximum stationary phase.

B. Growth in the Presence of Phage.—Bacteria grown in the presence of phage have failed to show any differences in their growth curves from those of broth cultures maintained under like conditions of temperature, pH, agitation, etc., except when the initial P/B ratio has been very high. That is, a normal growth curve is followed up to a certain point at which lysis begins explosively. There is no evidence that lysis is a continuous process from the time P and B are mixed or that B growth is stimulated by P. The special case of growth deviation seen in cultures containing very high $[P]$'s relative to $[B]$ at the start of growth is considered below.

C. Production of Phage Is Logarithmic.—In Figs. 2, 3, 4 and 5 the plot of log total $[P]$ against t is a straight line rising to a maximum and dropping off thereafter. Since the growth of bacteria is also logarithmic at first, it follows that the ratio P/B increases logarithmically with time. Fig. 6, containing the plots of four experiments, indicates that this is the case. One cannot say then that every time a B divides a certain amount of P is formed. If this were so the log plots of B and P against t would be parallel lines. Physically, the large value of $d \log P/d t$ as compared to $d \log B/d t$ signifies that the rate of P production is proportional to a power of the rate of B reproduction.

The curves of Figs. 2, 3, 4 and 5 show no conclusive evidence of a lag in P formation corresponding to the lag in B formation. Fig. 7 is a plot of two experiments performed particularly to detect a lag and both show very definitely a slight lag lasting between 0.4 and 0.5 hour. This would be overlooked in the ordinary run of experiments in which samples are taken at hourly intervals.

The view that P production is a process independent of bacterial lysis has been put forward by several investigators (3)–(13). Others, chief of whom is d'Herelle (19), feel that lysis is the essential part of bacteriophagy and that the accumulation of phage is dependent upon its liberation from lysing bacteria.

D. Relation of Extracellular P to Total P.—Figs. 2, 3, 4 and 5 contain plots of log extracellular $[P]$ against time. This represents the P content of the clear fluid left after centrifuging off B. Here again the

development of P is seen to be logarithmic, maintaining a constant ratio to the intracellular P as shown by the fact that the log plots of extracellular $[P]$ and total $[P]$ against t are parallel. Fig. 7 indicates that at 0.4 to 0.5 hour from the time of mixing P and B 75 per cent of the P present in the mixture is within or adsorbed to the B while but 25 per cent is free in the supernatant fluid. Other experiments of this sort give values of 70 to 90 per cent for the first and 10 to 30 per cent for the second of these fractions. After 0.5 hour

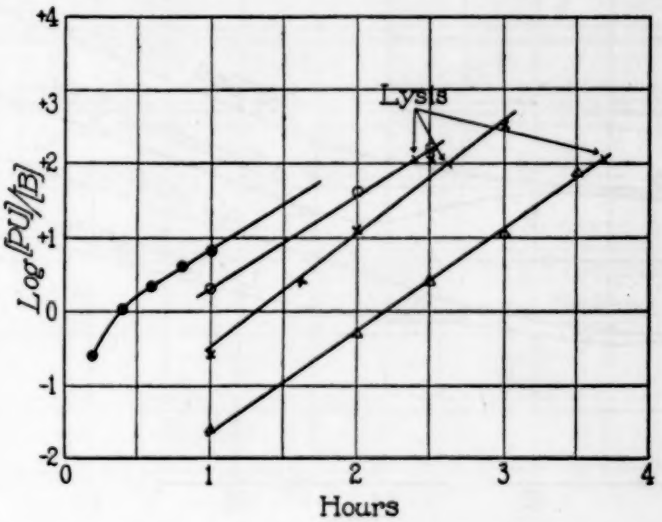

FIG. 6. The development of bacteriophage per organism with time in four separate experiments; *Bo* constant, *Po* varying. The plots show that the formation of P per B is a logarithmic process; also that lysis results when log $P/B = 2.1$.

and until the maximal $[P]$ is approached there appears to be an equilibrium between the P associated with the B and that free in the suspension, *i.e.*, the free $[P]$ is a constant low percentage of the total $[P]$. In this respect the P distribution is quite what one would expect in the case of a relatively simple chemical compound and not what would be anticipated if P is a comparatively complex organized body. As the total $[P]$ approaches a maximum (Figs. 2–5) the curve of extracellular $[P]$ flattens and the total $[P]$ curve descends from a maximum to meet it, indicating some destruction of intracellular P during lysis of B.

E. Growth of B Essential for Production of P.—The logarithmic production of *P* during the log phase of *B* growth suggested that *P* formation might be intimately associated with the growth of *B*. Several experiments have been undertaken to investigate this point.

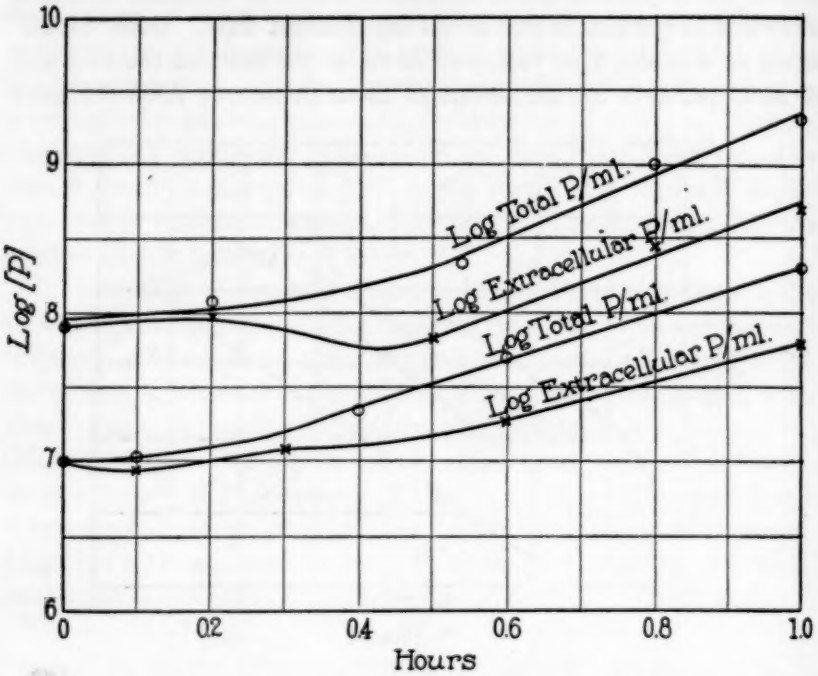

FIG. 7. Two experiments performed to detect lag in *P* formation and to establish the relationship between intracellular *P* and extracellular *P* early in the process of *P* production. A definite lag is evident, *P* production becoming logarithmic with *t* at 0.4 to 0.5 hour. By 0.5 to 0.6 hour the major percentage of total *P* has become intracellular *P*. The equilibrium between intracellular *P* and extracellular *P* established at this time is maintained up to $t_{(lysis)}$.

(*a*) *P Formation in P-B Mixtures with Initial* [*B*] *Approaching Be and with Initial* [*B*] > *Be.* *B* and *P* were mixed in broth in such amounts that the final respective concentrations were $1 \times 10^{9.05}$ *B*/ml. and $1 \times 10^{7.9}$ P.U./ml. The tubes were placed in the shaker at 36°C. and the *P* and *B* curves followed. The [*B*] was already near the limiting value of the maximum growth stationary phase (9.4) and the

growth curve (Fig. 8) showed merely a short phase of negative growth acceleration before reaching this [*B*], after which no growth was detectable. The [*P*] meanwhile had increased one hundred times but as growth ceased the curve of *P* production flattened also. No demonstrable lysis occurred in this suspension and no further increase in [*P*] was noted. Fig. 8 represents another experiment of the same sort. Here the initial log [*B*] was 9.7 (considerably > *Be*) and the corresponding log [*P*] 9.5. The mixture was kept at 36°C. in the shaker.

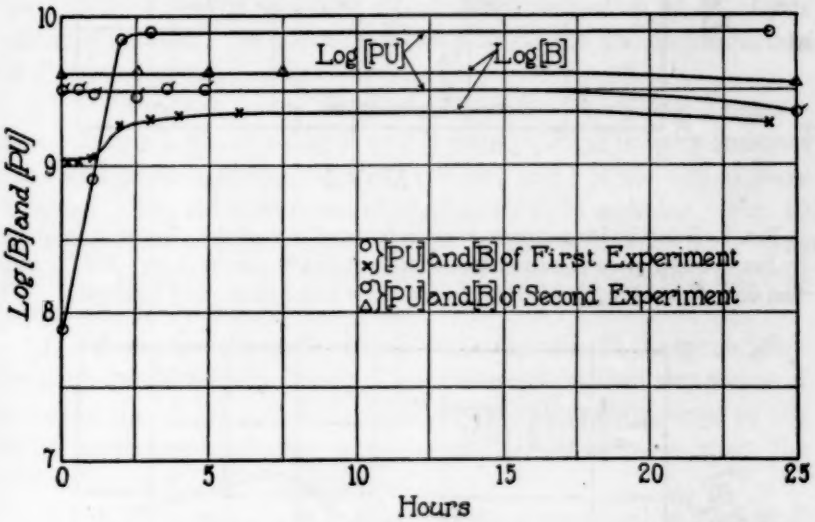

FIG. 8. Relationship between *P* formation and *B* growth. In Experiment 1 *Bo* is not far from *Be*, growth of *B* is limited and *P* production occurs only during the short period required for *Bo* to rise to *Be*. In Experiment 2 *Bo* > *Be*, there is no *B* growth and no *P* formation.

No growth of *B* took place and there was no production of *P* during 24 hours. That the cells were living was demonstrated by plate counts on controls at the end of the experiment.

(*b*) *P Formation in P-B Mixtures Made with Limited Supply of Nutrient Material and Kept at Moderately Low Temperature to Retard B Growth. Effect of Increasing Nutrients and Raising Temperature.*— Standard *P* was diluted 1–1,000 with saline. To 4 ml. of this dilution, *B* suspended in saline were added. The final concentrations were

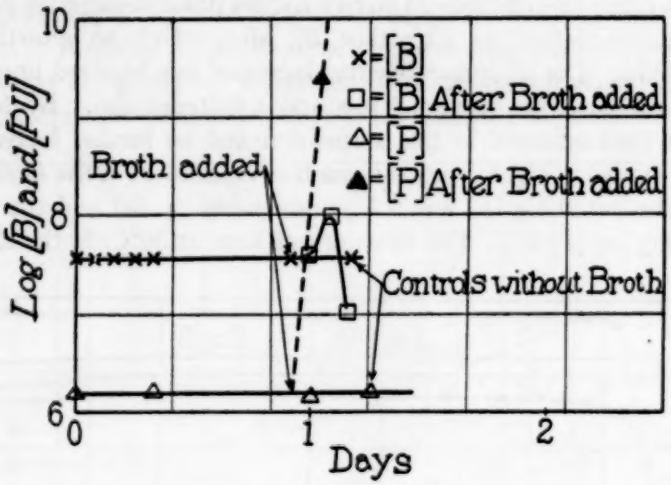

FIG. 9. Relationship between *P* formation and *B* growth. *B* growth inhibited by low concentration of nutrients and moderately low temperature. No *P* formation occurs until broth added and temperature raised permitting *B* growth.

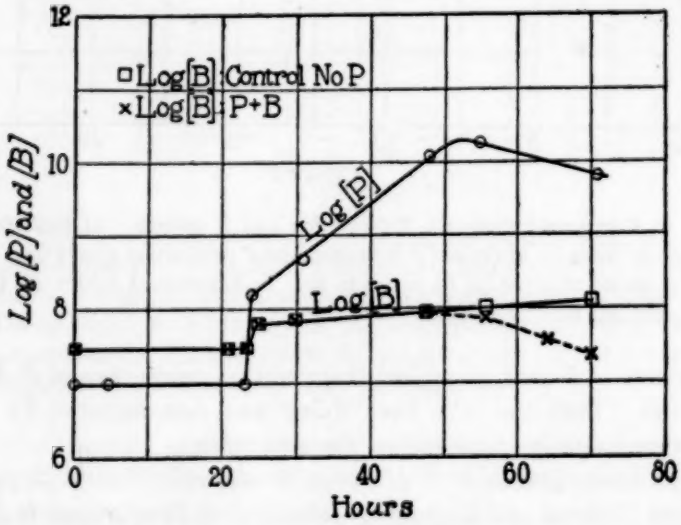

FIG. 10. Relationship between *P* formation and *B* growth. *B* growth retarded by low temperature (10°C.). *P* production proceeds only when [*B*] is increasing. Lysis occurs when log $P/B = 2.1$.

respectively $1 \times 10^{8.2}$ P.U./ml. and $1 \times 10^{7.4}$ *B*/ml. The amount of nutrient material was greatly reduced to inhibit growth of *B*; to further retard growth the tubes were kept at 21°C. Determinations of *P* and *B* at intervals indicated no rise in [*B*] or [*P*] up to 23 hours [Fig. 9). At this time 1 ml. of broth was added to each of several tubes and they were placed in the shaker at 36°C. As controls some of the original tubes without the addition of broth were shaken at 36°C. and others were kept at 21°C. The tubes receiving broth at once showed growth of *B* and the concomitant production of *P*. Lysis occurred as usual. In the control tubes no growth and no production of *P* were observed.

(*c*) *P Formation during Retarded B Growth at Very Low Temperature.*—Into a series of tubes *P* and *B* were pipetted in such amounts that final concentrations of 1×10^7 P.U./ml. and $1 \times 10^{7.4}$ *B*/ml. were attained. The tubes were maintained at 10°C. in a shaker. Fig. 10 indicates no bacterial growth for 22 hours. Likewise no *P* was formed. However, when *B* growth once began *P* production was also noted. Lysis eventually took place.

(*d*) *P Formation When Be Is Artificially Raised.*—If the assumption that *P* is produced only during *B* growth is correct then any means of inducing the *B* of a *P-B* mixture to grow beyond the level of the usual maximum stationary phase should result in more than the ordinary amounts of *P* being formed.

5 ml. of broth containing $1 \times 10^{9.4}$ *B*/ml. were added to 5 ml. of *P* containing 1×10^{10} P.U./ml. The mixture was dialyzed against broth through an acetic collodion membrane of such porosity that *B* and *P* were retained while metabolites and broth might be interchanged. A control of *B* in broth reached a value of $1 \times 10^{9.8}$ *B*/ml. before growth ceased. In the *P-B* mixture the *B* rose to $1 \times 10^{9.7}$/ml. and the *P* to $1 \times 10^{11.9}$ P.U./ml., a [P] some one hundred times that obtained in the usual lysate. Lysis occurred at this point.

In all these experiments then it was not possible to produce *P* without growth of *B*, indicating that *P* production is somehow conditioned by *B* growth. This intimate relationship between B growth and P production has been stressed in earlier work (4, 11, 14–18).

F. Lysis Occurs When Log P/B = 2.1 (Ratio P.U./*B = 125).*—So far in this discussion there has been no reference to the mechanism

initiating lysis of bacteria. Turning again to Figs. 2, 3, 4 and 5, it is evident that the decrease in $[B]$ begins when the total $[P]$ reaches a point 2.1 on the log scale above the maximal $[B]$. Assuming for the moment that this relationship is significant and that lysis will begin in any culture when the ratio of $P/B = 125$ or $\log P/B = 2.1$, it is obvious that those P-B mixtures in which the original $[P]$ is low com-

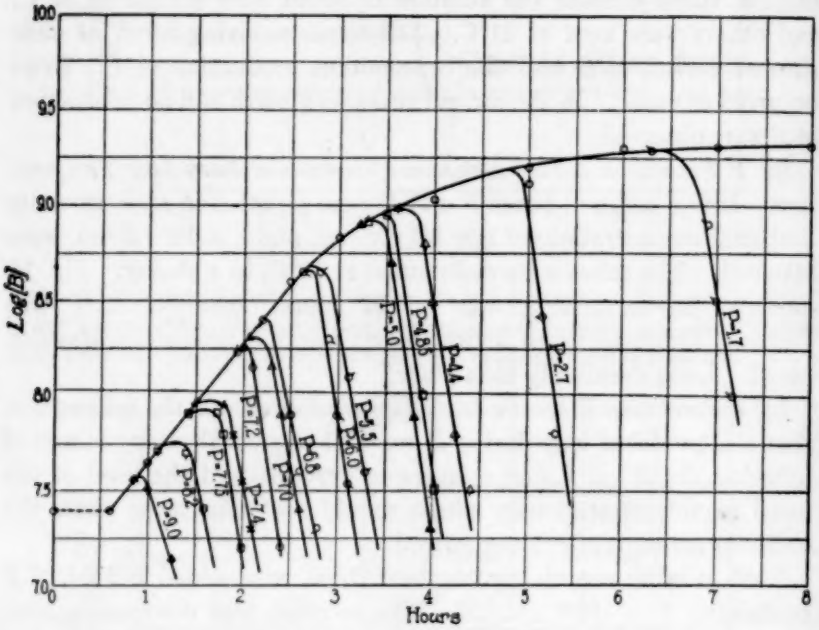

FIG. 11. B growth in broth and in P-B mixtures made with log $Bo = 7.4$ and various Po's (temperature 36°C.). The growth curves are identical until lysis begins. $t_{(lysis)}$ obviously depends upon Po. The process of lytic destruction of B is seen to be logarithmic with t and the values $-dB/dt$ are constant for widely varying Po's.

pared to $[B]$ will require a longer time to lyse than those having an original P/B ratio closer to the limiting value. This is experimentally true. The assumption will also explain the lack of growth or at most very slight growth seen in cultures to which have been added very high $[P]$'s as in the first curves of Fig. 11. Here the initial values of $\log P - \log B$ are $9-7.4$, $8.2 - 7.4$ and $7.75-7.4$ at the start of the

reaction. In the first instance lysis occurs very quickly for the ratio *Po/Bo* is near the critical value, in the second case there is slight growth until enough *P* has been produced to reach the limiting ratio and in the third instance even more bacterial growth is required.

So far then the assumption is qualitatively in accord with experimental facts. A further prediction is that any culture of bacteria will lyse without further growth of *B* if we add to it 125 P.U. for each *B*. The following experiments serve to substantiate the hypothesis and to

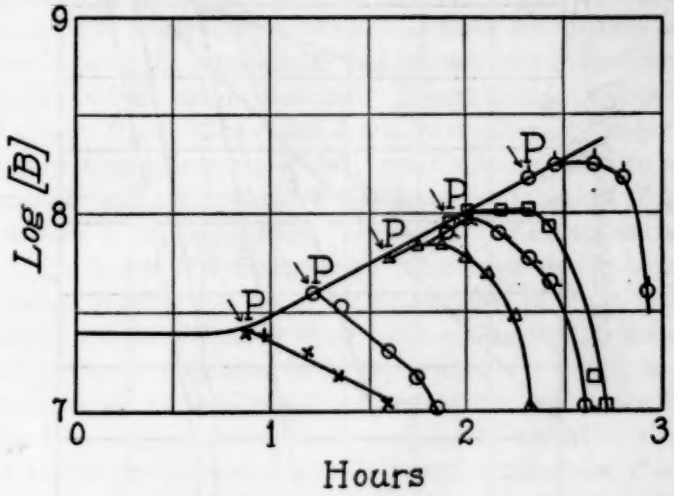

FIG. 12. Lysis is conditioned upon the value log $P/B = 2.1$. 4×10^{10} P.U. (vol. 4 ml.) were added to tubes of growing *B* (vol. 4 ml.) at double the [*B*]'s indicated (volume doubled by *P* addition and [*B*] halved). Lysis without growth occurred when log $P/B = 2.1$ or more. Below this value *B* growth proceeded until enough *P* was produced to reach the critical value.

stress the significance of the P/B ratio as an essential condition for the initiation of lysis.

(*a*) *Lysis without Further B Growth Follows the Addition of P to B in Amounts Effecting a P/B Ratio of 125 or More.*—4 ml. aliquots of *B* in broth (2.5×10^7 *B*/ml.) were grown at 36°C. in the shaker. At various points on the growth curve 4 ml. of *P* (titre 1×10^{10} P.U./ml.) were added to the *B*. The course of events after the addition of *P* was followed and the results plotted (Fig. 12). Lysis began imme-

diately in two instances, namely, when the P/B ratios at the time of P addition were 200 and 125. The addition of P at points higher than this on the $[B]$ curve did not result in immediate lysis; rather growth

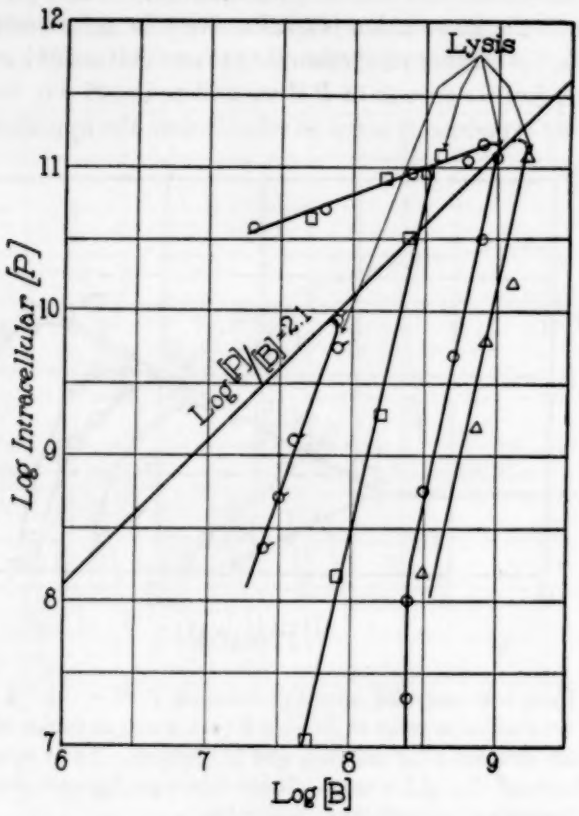

FIG. 13. Plot of log intracellular $[P]$ against log $[B]$. Lysis occurs when intracellular $[P]/[B] = 2.1$. This value holds for the intracellular P/B ratio as well as for total P/B since intracellular P constitutes the major percentage of total P.

continued with the concomitant production of P until enough was formed to attain the critical P/B ratio.

(*b*) *Lysis at Low Temperature Occurs When Log P = Log B + 2.1.*— The experiments done at 10°C. show the same general trend, i.e., a P/B ratio of approximately 125 must be reached before lysis begins (Fig. 10).

The experimental data point definitely then to the attainment of a particular [*P*] per *B*, and not to a critical [*P*] per ml., as the condition responsible for the lytic process. It cannot be said at this time, however, whether the ratio intracellular *P*/*B*, extracellular *P*/*B* or total *P*/*B* is the one involved. If a series of *P*-*B* mixtures are centrifuged at intervals shortly after the addition of *P* to *B* it is possible to separate the cells from *P* at various stages of the process of *P* diffusion into (or adsorption to) *B*. By observing the $t_{(lysis)}$ of such bacteria resuspended in broth and comparing with controls resuspended in the supernatant *P* it might be expected that some information as to the relative rôles of the intracellular and extracellular *P* fractions in the instigation of lysis could be elicited. Experimentally, the cells separated shortly after mixing *P* and *B* require considerably longer for the onset of lysis than those centrifuged from *P* when equilibrium between intracellular and extracellular *P* has become established (0.5 hour). The removal of the extracellular *P* after this time causes no change in $t_{(lysis)}$. Further, if *P*-*B* mixtures are centrifuged shortly before lysis is expected to occur and the *B* are resuspended in broth, lysis commences at the same time in these tubes and in control suspensions centrifuged and resuspended in the supernatant *P*.

This all might be interpreted as stressing the importance of intracellular *P* in bringing about *B* lysis were it not for the following facts: When equilibrium between intracellular and extracellular *P* once has been attained the value of extracellular [*P*] is very small compared to intracellular [*P*]. Therefore any difference in $t_{(lysis)}$ occasioned by its removal is quite small and probably is within the limits of experimental error. Also, shortly after resuspending the centrifuged cells in broth at any time after intracellular-extracellular *P* equilibrium has become established the extracellular fraction quickly appears again and equilibrium is maintained up to $t_{(lysis)}$. The significance of the longer $t_{(lysis)}$ noted in the case of cells centrifuged and resuspended in broth soon after the addition of *P* to *B* is likewise open to question. It is known that but a small percentage of total [*P*] comes down with the *B* at this time; again, extracellular *P* is present immediately after the *B* are resuspended in broth. Consequently the difference in $t_{(lysis)}$ may well be due to the lower value of total [*P*] or extracellular [*P*] and not to the smaller intracellular [*P*].

Obviously this type of experiment leads to no definite conclusions beyond the one established point that lysis follows the attainment of a particular P/B ratio. Empirically and rationally any one of the ratios intracellular $[P]/[B]$, extracellular $[P]/[B]$, or total $[P]/[B]$ might represent the prime conditioning agent for lysis.

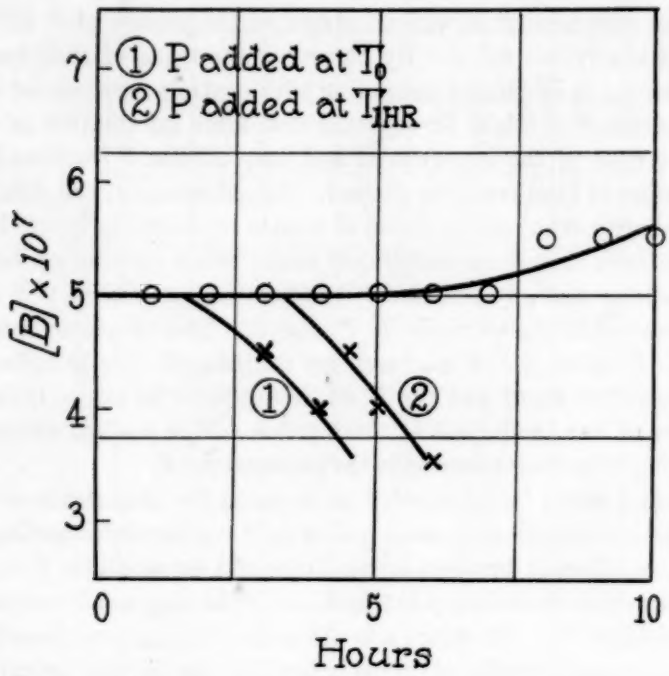

FIG. 14. *B* growth is not essential for lysis. *P* added to *B* during prolonged lag phase of *B* growth induced by low temperature (12°C.). Log P/B = 2.2 in both instances. Lysis occurred before *B* growth became evident in controls of *P-B* mixtures.

G. Growth of B Is Not Essential for Lysis.—The question of whether or not *B* must be in the growth phase for lysis to occur is a difficult point to investigate experimentally. *B* which have reached the maximum stationary phase cannot be used because the addition of *P* dilutes the suspension below the maximum $[B]$ and growth begins again. If the *B* are centrifuged from a fully grown culture and are

made up to the maximal stationary [*B*] in *P*, the [*P*] attainable will still be below the critical *P*/*B* ratio because log [*P*] of standard undiluted *P* is only 10.0 and *Be* = 9.4. Consequently, one is forced to inhibit growth by some artificial means, chemical or otherwise. It seemed best in the present case to avoid complications inherent in the addition of chemicals to the *P*-*B* system and rather to make use of the fact that the lag phase of *B* growth at low temperatures is very markedly prolonged.

(*a*) *B Can Undergo Lysis during the Lag Phase Before Growth Begins.*—Tubes containing a total of 25×10^7 *B* in 1 ml. of broth were placed in the water bath shaker at 12°C. At various times 4 ml. portions of *P* (titre 1×10^{10} P.U./ml.) were added to the *B* suspensions, giving a *P*/*B* ratio of 160. Controls of *B* alone without *P*, both concentrated and dilute, were run at the same time. The *B* curves are given in Fig. 14 and indicate quite clearly that lysis had started before growth began.

From this experiment and the preceding ones it seems justifiable to conclude that the essential condition for initiation of lysis is a minimum *P*/*B* ratio of 125 and that *B* need not be growing for lysis to occur. This conclusion is made more tenable by the fact that lysis of cultures seeded with very small *Po's* occurs when the growth curve is definitely well within the maximal stationary phase (Fig. 11) (a common observation).

H. Lytic Destruction of B Is a Logarithmic Process.—From the graphic representation of [*B*] during lysis of a *B* suspension (Fig. 11) by various initial [*P*]'s it is apparent that *B* destruction is logarithmic with time, in this respect being analogous to most death rate processes. Further, the rate at which lysis proceeds with a given initial [*B*] is constant for widely varied *Po*'s. The temperature coefficient of the rate of lysis is remarkably high (compare Figs. 10 and 11).

I. The Major Portion of P Present at End of Bacteriophagy Is Set Free from Lysing Bacteria.—The plots of Figs. 2, 3, 4 and 5 are logarithmic and give a false impression of the conditions actually obtaining during lysis. Fig. 15 represents two sets of the same data plotted on an arithmetic scale. The lower points are of course meaningless because of the large scale used, but the curves during the time of lysis show clearly that 30 to 50 per cent of the maximal total [*P*] or 40 to

60 per cent of the maximal intracellular [*P*] formed in the course of the reaction are lost as the *B* are destroyed. Since at the maximum [*P*]

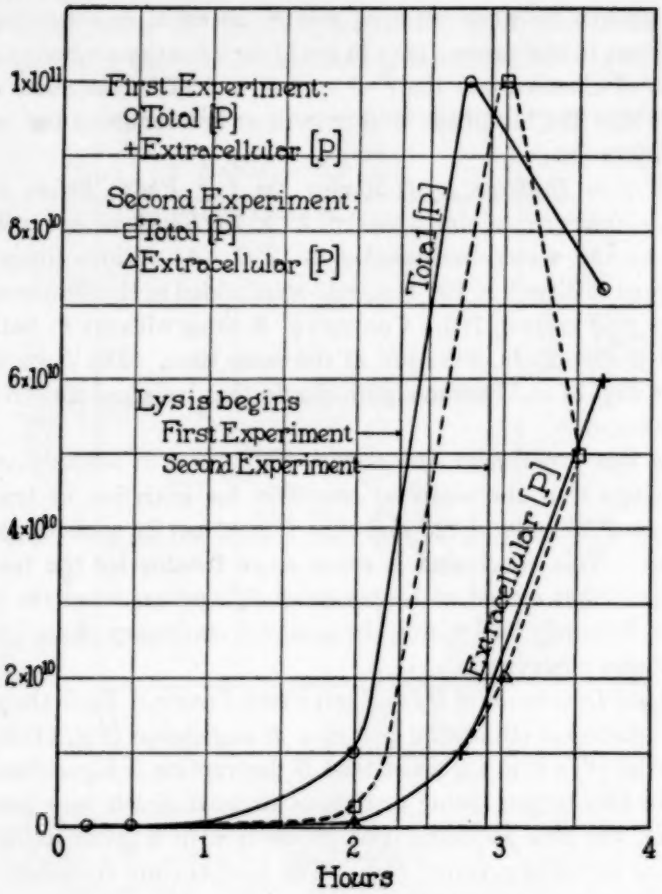

FIG. 15. An arithmetic plot of total *P* and extracellular *P* against *t* in two different *P*-*B* mixtures to show relationship existing at the development of maximal [*P*] and $t_{(\text{lysis})}$.

attained, intracellular *P* constitutes the major percentage of total [*P*], it is probable that the chief loss in *P* during lysis takes place in the intracellular fraction.

J. End Titre Values after Bacteriophagy.—When the entire process of bacteriophagy is completed the lysate is left with a certain [*P*] determined by the now completed *P-B* reaction and maintained at a constant level for long periods of time if the phage is stored at low temperatures. These final titre values for various initial values of [*B*] and [*P*] fall within a relatively narrow range. The explanation of this lies in the large value of $d\,P/d\,t$ as compared to that of $d\,B/d\,t$. Fig. 16 is a plot of *B* growth against time and with it are curves of *P*

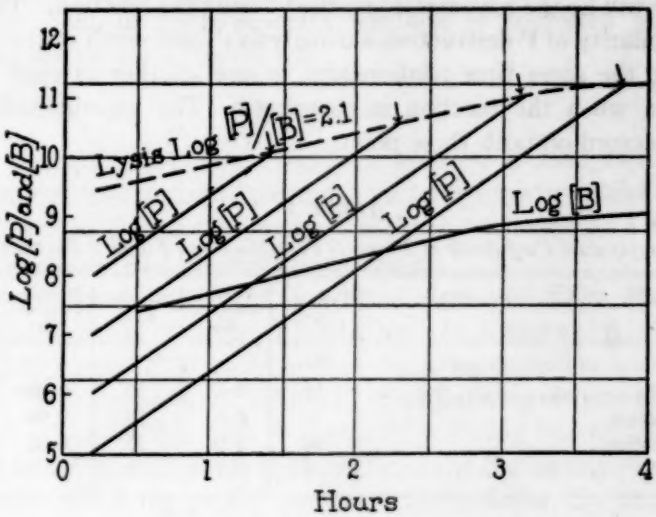

FIG. 16. A plot of log [*B*] against *t*, with plots of log [*P*] against *t*. The latter curves originate in various *Po*'s and are assigned an average slope. The breaks at the origin allow for average lags in *P* and *B* development. Lysis is seen to occur when log [*P*]/[*B*] = 2.1. $t_{(lysis)}$ is predicted fairly accurately (*cf.* Fig. 11).

formation to which have been assigned the average slope of numerous experimental curves. Clearly, on all the log [*P*] curves originating in widely varying *Po*'s the points at which log [*P*] = 2.1 + log *B* (lysis begins) lie within a narrow zone of log [*P*] values (10–11). That is, the maximal [*P*]'s resulting from the reaction of a constant *Bo* with different *Po*'s theoretically should vary only some ten times in titre.

Figs. 2, 3, 4 and 5 confirm this prediction and numerous other

experiments show the same general grouping of maximal [*P*] values. The destruction of *P* during lysis is sufficiently regular to maintain this relationship in the lysates after completion of bacteriophagy.

Fig. 16 also predicts the time for lysis with reasonable accuracy (compare with Fig. 11) and brings out an additional point, namely, that for a series of *P-B* mixtures made with constant initial [*B*] and progressively larger *Po*'s the maximal [*P*]'s will be in the reverse order of magnitude of the initial [*P*]'s. That is, the larger the *Po* the smaller will be the maximal [*P*] reached during the reaction. Further, the regularity of P destruction during lysis should result in the lysates bearing the same titre relationships to one another as exist at the maxima when the reaction is completed. The experimental facts are in accord on both these points.

TABLE I

Temperature Coefficients of P and B Formation and P and B Destruction

	Temperature$_1$	Velocity constant K_1	Temperature$_2$	Velocity constant K_2	Q_{10}
	°C.		°C.		
B growth	36	0.6	10	0.006	5.9
P production	36	1.6	10	0.06	3.5
B destruction	36	3.0	10	0.02	6.9
P "	36	0.75	10	0.025	3.7

K. The Temperature Coefficients of P and B Formation and P and B Destruction.—From the experiments already performed (Figs. 2, 3, 4, 5, 10 and 11) it is possible to get some idea of the temperature coefficients of the various processes included under "bacteriophagy." Table I lists the essential data.

These figures bring out again the fact that the *P-B* system cannot be analyzed from the viewpoint of a single reaction. The temperature coefficients for *P* and *B* formation differ significantly and in very nearly the same ratio as those for *P* and *B* destruction differ. *P* formation has been shown to be conditioned by *B* reproduction but granting this intimate relationship, the difference in temperature coefficients of the two processes suggests that *B* growth and *P* formation are not one and the same reaction. *P* cannot be formed unless the *B* are

growing but once *B* growth has begun, and assuming that it continues, the reaction responsible for *P* production progresses as a concomitant process.

In just this same sense *P* inactivation during lytic destruction of *B* is conditioned upon *B* dissolution. The simplest theoretical case possible would be the building up of an unstable *P-B* system which would go to pieces *in toto;* then for every *B* lost, a certain number of P.U. would disappear. Experimentally this is true only in so far as the processes of *P* inactivation and *B* destruction are concomitant. However, that they are separate reactions is suggested by their different temperature coefficients.

DISCUSSION

The essential points in the reaction between 16 hour cultures of staphylococci and antistaphylococcus bacteriophage in nutrient broth of pH 7.6 at 36°C. and with mechanical shaking to maintain a uniform *B* suspension may be summarized as follows:

Bacterial growth in the *P-B* mixture does not differ from the growth of identical controls without *P* until such time as lysis begins, unless the initial *P/B* ratio is very high, an exception considered below. Both *B* growth and *P* formation exhibit a lag of approximately 0.5 hour. During this time *P* is rapidly diffusing into or becoming adsorbed to *B* at such a rate that by the end of the lag period only 10 to 30 per cent of the total *P* present is extracellular, the remainder being associated with the *B*. As the *B* growth curve enters the short phase of positive growth acceleration, equilibrium between the intracellular and extracellular *P* fractions becomes established and is maintained up to the time of lysis, extracellular [*P*] representing a small constant percentage of total [*P*]. This distribution of *P* on a constant percentage basis is suggestive of the manner in which a relatively simple chemical compound would be distributed and is not at all typical of the distribution one would expect if bacteriophage is a comparatively complex substance.

All experiments performed to investigate the relationship between *B* and *P* indicate that no *P* is produced without *B* growth, thus establishing the latter process as an essential conditioning factor for *P* formation. During the period of logarithmic *B* growth *P* formation

is likewise logarithmic but proceeds at a much faster rate. That is, the rate of P production is proportional to a power of the rate of B reproduction, making untenable the usual statement that each time a B divides a certain amount of P is formed.

When the value of log P/B = 2.1 (P/B = 125) lysis occurs. This statement would be true, with different numerical values of course, for log intracellular P/B, log extracellular P/B or, as written, log total P/B. That is, there is no conclusive experimental evidence at this time as to which P fraction constitutes the essential conditioning agent for lysis. The most one can say is that lysis is not brought about by the attainment of a particular [P] per ml. but by a certain [P] per B. Obviously, this limiting value for any initial [B] is reached sooner the higher the initial [P]. When log P/B at the time of mixing is already above 2.1 there is no growth of B and lysis soon occurs. The large value $d\ P/d\ t$ as compared to that of $d\ B/d\ t$ renders clear the experimental fact that the maximal [P]'s attained during the reaction of a constant [B] with various [P]'s fall within a very narrow range, those having been made with high initial [P] having a lower maximal [P] value than those made with low Po.

The lytic destruction of B is again logarithmic with time just as are most death rate processes and the value $-d \log B/d\ t$ for a particular initial [B] is constant for various Po's. There is good evidence that cells need not be growing in order to undergo lysis. As B are lysed a considerable percentage of the total maximal P formed is destroyed, the chief loss probably occurring in the intracellular fraction. The destruction of P is sufficiently regular to maintain the relationship established at the maxima. That is, lysates made by mixing a constant Bo with various Po's vary only some ten times in [P] after lysis is completed. Just as in the case of the maxima, the final [P]'s vary in inverse order of the Po's. The major portion (70 to 90 per cent) of the final P present after the completion of bacteriophagy is set free during the short period of bacterial dissolution.

There is a marked difference in the temperature coefficients of P and B formation. Further, the temperature coefficients of P and B destruction during lysis differ in almost the same ratio. These data suggest that while B growth may be an essential condition for P formation the two processes are basically separate reactions. A

similar interpretation would hold in the case of B dissolution and P inactivation.

Agitation of test series is a necessary condition in experiments of the sort described in this paper. Without mechanical shaking of the tubes B settle out. As an example of what may follow it is obvious that the critical P/B ratio for lysis can be reached in the relatively sparse B population of the supernatant and the $[B]$ considerably depleted by lytic action, whereas if measures are taken to maintain a uniform $[B]$ such a sequence does not occur. Anomalous results of this sort have been observed whenever shaking has been omitted.

The Kinetics of Bacteriophage Action

(*a*) *The Fundamental Relationship between B Reproduction and P Formation during the Logarithmic Phase of B Growth.*—The following equation expresses the relationship between P formation and B growth during the logarithmic phase:

$$P/Po = (B/Bo)^n \tag{1}$$

where P = Phage/ml.
and B = Bact./ml.

$$\therefore P = Po \frac{B^n}{B_o^n} \tag{2}$$

of which the differential form is:

$$dP/dB = Po \frac{nB^{n-1}}{B_o^n} \tag{3}$$

From (2)

$$P/B = Po \frac{B^{n-1}}{B_o^n} \tag{4}$$

The obvious step from this point is to solve Equation (8) below for B in terms of Bo, Be and t. Since it is experimentally true that lysis occurs when $\log P/B = 2.1$, substitution for B from Equation (8) in the right hand term of Equation (4) would make possible a direct solution for $t_{(\text{lysis})}$. Practically, however, it is simpler to effect this

solution for t in two steps, first obtaining B (at lysis) from (4), substituting it in Equation (8) and solving the latter for $t_{(\text{lysis})}$.

(*b*) *Determination of Log B at Lysis.*—From Equation (4) it follows that:

$$\text{Log } (P/B) = \log Po + (n - 1) \log B - n \log Bo$$

At lysis $\log P/B = 2.1$.

$$\therefore \text{Log } B_{(\text{at lysis})} = \frac{2.1 + n \log Bo - \log Po}{n - 1} \tag{5}$$

(*c*) *General Relationship between Po and* $t_{(\text{lysis})}$.—The plot of log B against t is similar to the curve of an ordinary autocatalytic reaction. If it is assumed that:

$$dB/dt = gB\,(Be - B) \tag{6}$$

where Be is $[B]$ at the maximum stationary growth phase and B the $[B]$ at any time, it follows that:

$$dB/B\,(Be - B) = g\,dt$$

$$\therefore \int dB/B\,(Be - B) = \int g\,dt$$

or

$$-\frac{1}{Be} \log_e \frac{Be - B}{B} = gt + k$$

$$\therefore t = -\frac{1}{gBe} \log_e \frac{Be - B}{B} - \frac{k}{g} \tag{7}$$

Evaluation of k: When $t = 0$, $B = Bo$

$$\therefore -\frac{1}{gBe} \log_e \frac{Be - Bo}{Bo} - \frac{k}{g} = 0$$

$$\frac{k}{g} = -\frac{1}{gBe} \log_e \frac{Be - Bo}{Bo}$$

and

$$k = -\frac{1}{Be} \log_e \frac{Be - Bo}{Bo}$$

Evaluation of gBe: From equation (6)

$$dB/B\,dt = g\,(Be - B)$$

But

$$d \log_e B/d\,B = \frac{1}{B}$$

$$\therefore\ d \log_e B/d\,B \cdot d\,B/dt = g\,(Be - B)$$

Hence

$$d \log_e B/dt = g\,(Be - B)$$

When

$$B << Be,\ (Be - B) = Be$$

$$\therefore\ d \log_e B/dt = g\,Be$$

From Fig. 11, the plot of $\log_{10} B$ against t, $d \log_{10} B/dt = 0.6$

$$\therefore\ gBe = (2.3)\ (0.6)$$

and

$$\frac{1}{gBe} = \frac{1}{(2.3)\ (0.6)}$$

Equation (7) then becomes:

$$t = -\frac{2.3}{(2.3)\ (0.6)} \log_{10} \frac{Be - B}{B} + \frac{2.3}{(2.3)\ (0.6)} \log_{10} \frac{Be - Bo}{Bo}$$

$$= 1.7 \left[\log_{10} \frac{Be - Bo}{Bo} - \log_{10} \frac{Be - B}{B} \right]$$

$$= 1.7 \left[\log_{10} \frac{B\,(Be - Bo)}{Bo(Be - B)} \right] \qquad (8)$$

Table II lists the values of B at lysis calculated fromEquation (5) and the values for $t_{(\text{lysis})}$ obtained by substituting the calculated $[B]$'s in Equation (8).

The values of $[B]$ at lysis and t for lysis calculated from the two equations compare well with the observed figures over a range of

initial [*P*] values varying 100,000 times. Below log *Po* = 4.0 agreement is not good for two reasons; first, because Equation (7) becomes extremely sensitive to the value assigned to *Be* and second, because Equation (5) behaves similarly to values of *n*. However, Equation (7) predicts the experimental fact that with very low values of *Po* lysis

TABLE II

[*B*] at lysis, observed and calculated from

$$\text{Log } B = \frac{2.1 + n \log Bo - \log Po}{n - 1}$$

$t_{(\text{lysis})}$, observed and calculated by substitution of the value of log *B*

$$\text{in } t = \frac{1}{g\,Be} \log \frac{B\,(Be - Bo)}{Bo\,(Be - B)}$$

$$\frac{1}{g\,Be} = 1.7 \qquad n = 4$$

Log *Po*	Log *Bo*	[*B*] at lysis		*t* (−0.5) for lysis	
		Calculated	Observed	Calculated	Observed
9.0	7.4	7.6	7.6	0.30	0.40
8.2	7.4	7.8	7.75	0.70	0.70
7.75	7.4	8.0	7.90	1.04	0.95
7.20	7.4	8.2	8.25	1.40	1.45
7.0	7.4	8.25	8.30	1.50	1.60
6.4	7.4	8.43	8.50	1.84	1.90
6.0	7.4	8.6	8.65	2.16	2.20
5.0	7.4	8.9	8.9	2.86	2.80
4.85	7.4	8.95	8.95	3.00	2.95
4.40	7.4	9.10	9.00	3.13	3.10
4.00	7.4	9.20	9.10	3.98	3.75

(t − 0.5 hr.) is used to allow for the lag of 0.5 hr. in *B* growth.

will never occur. That is, as *B* becomes large (*Be* − *B*) becomes progressively smaller until finally it has a value of zero. Solution for *t* is then indeterminate.

(*d*) *Relationship between Log Po and* $T_{(\text{lysis})}$ *for a Given Value of Bo during Logarithmic Growth Period.*–From Equation (5) at lysis

$$2.1 = \log Po + (n - 1) \log B - n \log Bo$$

During the logarithmic phase of B growth

$$B = Bo\, e^{gt}$$

or

$$\text{Log } B = \log Bo + (0.43)\, gt$$

Substituting this last value for log B in (5):

$$2.1 = \log Po + (n - 1)\,(\log Bo + 0.43\, gt) - n \log Bo$$

or

$$t = \frac{2.1 - (n - 1) \log Bo - \log Po + n \log Bo}{(n - 1)\,(0.43)\, g}$$

$$= \frac{9.5 - \log Po}{1.7} \qquad (9)$$

Equation (9) applies numerically to the value log $Bo = 7.4$

TABLE III

t_{lysis} Observed and Calculated from Equation (9).

Log Po	Calculated ($t_{(lysis)} - 0.5$) $9.5 - \log Po/1.7$	Observed ($t_{(lysis)} - 0.5$)
7.0	1.47	1.5
6.0	2.06	2.2
5.0	2.65	2.7

(t − 0.5 hr.) is used to allow for the lag of 0.5 hr. in B growth.

Equation (9) predicts that with a constant initial Bo the plot of $t_{(lysis)}$ against the log Po values used, will be a straight line. The kinetic analysis of the P-B reaction thus rationalizes the method employed for quantitative P determinations (*q. v. i.* under "Methods") (2), a procedure developed originally on the basis of experimental observation alone. While Equation (9) deals with t values for the beginning of the lytic process and the t values utilized in the P determinations represent the time periods required to reduce the B suspension to a particular turbidity end-point, there is no discrepancy involved in applying the general relationship between Po and $t_{(lysis)}$ denoted in Equation (9) to the analytical method. Practically, the reduction of the heavy B suspension to a certain [B] end-point is much

to be preferred because the latter is technically convenient and decidedly more accurate than is the fixation of [*B*] at the beginning of lysis. The *t* values so obtained give just the same type of plot against log *Po*'s as do the [*B*]'s at the start of lysis. That is, for any *Po* the time consumed in reducing [*B*] at the start of lysis to the [*B*] used as an end-point is practically constant within the range used for *P* determinations.

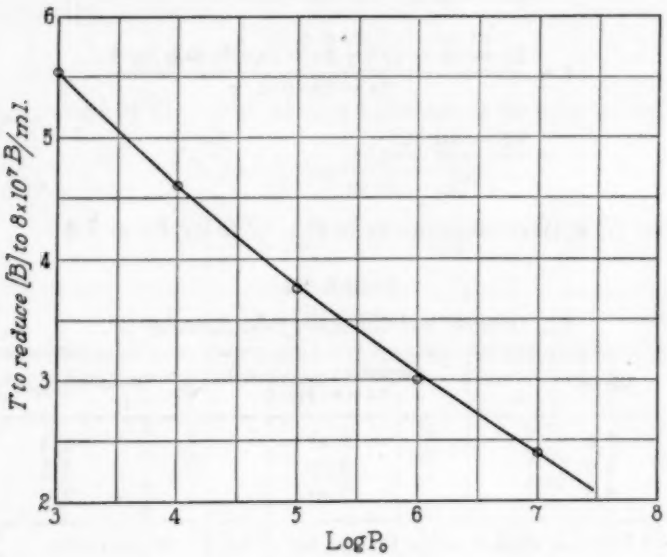

FIG. 17. Relationship between *Po* and *t* required to reduce [*B*] to a particular turbidity end-point. This curve is used in the method for determining *P* quantitatively.

It is also clear that theoretically the end-points given by log *Po* values < 5.0 should not lie on the standard plot of log *Po* against *t* (a straight line), for *B* growth in such cases runs beyond the logarithmic phase into the phase of negative growth acceleration. Experimentally this is found to be true and in making *P* determinations the standard log *Po* values used are 7.0, 6.0 and 5.0. The curve can of course be extended to log $Po = 4.0$ or even 3.0 but the points obtained will lie on a curve as indicated in Fig. 17. In the original description of the method for quantitative *P* determinations (2) the values of

standard log *Po*'s were 6.0, 5.0 and 4.0. The plot of these values against t (to reduce the $[B]$ to a certain turbidity end-point) was shown as a straight line. Actually from log $Po = 5.0$ on down it begins to curve slightly although practically no great difference is made in considering it a straight line for carrying out P determinations (*cf.* Fig. 17).

Methods

Method of Determining Bacteria.—Routinely, cell concentrations were determined by comparing the unknown with turbidity standards. The latter were prepared each day from cultures of the staphylococcus being studied and readings were made under conditions giving a maximum Tyndall effect (1). In the range from 3×10^7 B/ml. to 15×10^7 B/ml. determinations can be made to within ± 5 per cent, a degree of accuracy entirely sufficient for present purposes. With higher $[B]$'s the unknowns were always diluted with broth so as to fall within these limits The centrifuged sediment method (1) was employed regularly to check the turbidity estimates.

In the few instances requiring determination of living cell numbers the usual plate technic was followed. It will be noted in Fig. 1 that the living cell count as determined by plating is always slightly less than the total cell count as estimated by turbidity or centrifuged sediment measurements. This difference is probably due in part to the clumping together of cells, causing groups to appear as single colonies on the plate and to the fact that some cells of such preparations are not viable.

Method of Determining Bacteriophage.—The procedure employed is based upon the experimental observation that the time required to reduce a set concentration of growing phage-susceptible bacteria contained in a unit volume of P-B mixture to an arbitrary turbidity end-point is a function of the amount of phage present at the start of the reaction. A detailed description of the method is given in a recent paper (2). The accuracy of phage determinations made by this procedure is ± 3 per cent in the range of standard P dilutions from 10^{-3} to 10^{-5} (corresponding to 10^7 to 10^5 P.U./ml.). In the present work P determinations were made on samples taken at various time intervals. Each unknown was at once diluted serially by tenths and 4 ml. aliquots of appropriate dilutions were mixed with 1 ml. portions of a freshly made staphylococcus suspension in broth containing 12.5×10^7 B/ml.

The P-B mixtures were placed in the water bath shaker at 36°C. Heavy growth occurred in all tubes except those made with P of very high titre. Such tubes were disregarded. Tubes containing from 1×10^5 to 1×10^7 P.U./ml. furnished at some time between 2 and 4 hours a sharp decrement in B for which the curve of $[B]$ against time could be accurately fixed by turbidity readings at intervals of 0.1 hour when lysis was definitely under way and $[B]$ was $< 20 \times 10^7$/ml. On the plot of $[B]$ against time 8×10 B/ml. was chosen as an end-point and the time

required for the lysing suspension to reach this value was read directly. Referring to the control plot of log P.U. against the time to reach the same end-point, the log P.U. corresponding to the time figure of the unknown was determined. Converting to antilogs and multiplying by the dilution gave the value of the unknown in P.U. This value was automatically checked in nearly all instances by the fact that two or three tubes would give readings in the range from 1×10^5 to 1×10^7 P.U./ml.

The staphylococcus suspensions used throughout a day's work were prepared from a single saline suspension made early in the day as described under "Set-up of kinetic experiments." The cell content of the latter was accurately known (centrifuged sediment method, ±2 per cent), any required cell concentration could be made up readily, and control experiments showed no appreciable change in the numbers of bacteria present over a period of 8 hours. Likewise no qualitative alterations in the organisms were demonstrable. Thus, a control curve for P determinations made early in the day with B from a particular suspension could be matched closely with known P dilutions and the same B at any time during the day. This point is of importance since P estimations were made as much as 7 hours after the first control set was started. In order to rule out adequately any possible difficulty on this score two or three control sets of known $[P]$'s were run at various times during each series of P determinations.

Set-Up of Kinetic Experiments.—Unless otherwise noted all experiments were run as follows: A 16 hour culture of *S. aureus* grown in a Blake flask was suspended in saline and the mixture filtered through a Schleicher and Schüll Faltenfilter No. 588. The cell content of the suspension was determined by the centrifuged sediment method. Dilutions in broth were prepared and the bacteria immediately added to appropriate concentrations of phage contained in a series of tubes; controls without P were of course always included. Volume was kept constant at 5 ml. throughout. The tubes were placed in a shaker mounted in a water bath maintained at 36°C. At required time intervals tubes were removed for estimating the concentration of B and for making P determinations. After samples were pipetted off such tubes were discarded. Usually P determinations were made for total P/ml. and for extracellular P/ml. In the former case the contents of the tube were diluted by tenths in broth and 4 ml. aliquots of two or three serial dilutions were mixed with 1 ml. of a B suspension in broth made at this same time. The procedure outlined above was followed as to temperature, shaking, turbidity readings and calculations.

As a rule the B present in the unknown did not interfere with the P determination. This is quite what one would expect, for an average sample is diluted at least to 10^{-2} or 10^{-3} for analysis and the $[B]$ required to yield barely visible turbidity is 1×10^7 B/ml. Hence a suspension to interfere in this way must contain 1×10^{10} B/ml., a $[B]$ four times the maximal value ordinarily encountered. The small residual $[B]$'s left in the dilutions usually used were negligible when compared to the 2.5×10^7 B/ml. added for the P determination.

For the estimation of extracellular phage, a term employed merely to designate that portion of the total lytic agent not in or adsorbed to bacteria, the sample was centrifuged at 2600 R.P.M. for 0.1 hour immediately after removal from the bath. There is an obvious inaccuracy here since the separation of *B* from the supernatant is only partial at best. However, the number of *B* remaining in suspension was shown by control experiments to constitute a negligible percentage of the *B* thrown down and no appreciable error was introduced by considering the supernatant as *B*-free unless samples were allowed to stand a considerable length of time.

The separation of *B* and *P* is a gradual process and the reaction between them does not stop during the interval required for fairly complete sedimentation. Consequently, as a mean, the middle of the time interval required for centrifuging was used as the sampling time. Dilutions of the supernatant were run along with the total *P* determinations and in an identical manner.

SUMMARY AND CONCLUSIONS*

The above data relating to the reaction between 16 hour cultures of *S. aureus* and antistaphylococcus bacteriophage in nutrient broth of pH 7.6 at 36°C. and with mechanical shaking to maintain a uniform *B* suspension, bring out the following points:

(a) *B* growth in *P-B* mixtures does not differ from growth in controls without *P* except in the case of a very high initial *P/B* ratio as noted below. There is no evidence that lytic destruction of *B* begins shortly after mixing *P* and *B* nor that *B* growth is stimulated by *P*, for the *B* growth curves in the presence of ordinary [*P*]'s and in controls are identical. Only at the sudden onset of the rapid lytic process does the *B* curve of a *P-B* mixture deviate from the control curve.

(b) *B* growth is an essential conditioning factor for *P* formation.

(c) Both *B* growth and *P* production exhibit short lags. During this time *P* diffuses into or becomes adsorbed to *B* so rapidly that by the end of the lag period only 10 to 30 per cent of the total *P* present is extracellular, the remainder being associated with the *B*.

(d) During the logarithmic *B* growth phase, *P* formation is also logarithmic but proceeds at a much faster rate. That is, $d\,P/d\,t$ is

* Symbols used:

B = Bacterium
P = Bacteriophage
[*B*] = Bacteria / ml.
[*P*] = Phage / ml.
Bo = Initial value of [*B*]
Be = [*B*] at maximal stationary phase of *B* growth
Po = Initial value of [*P*]

proportional to a power of $d\ B/d\ t$. Consequently the statement that each time a B divides a certain amount of P is formed is not correct.

(e) As B growth enters the phase of positive acceleration equilibrium between the extracellular and intracellular P fractions becomes established and is maintained up to the onset of lysis, extracellular $[P]$ representing a small constant percentage of total $[P]$. The distribution of P on a constant percentage basis suggests the manner in which a relatively simple chemical compound would be distributed and is not at all typical of the distribution one would expect if P were a complex organized parasite.

(f) When the value of $\log P/B = 2.1$ lysis begins. Obviously, this limiting value for any initial $[B]$ is reached sooner the higher the initial $[P]$. When $\log P/B$ at the time of mixing P and B is already 2.1 or greater, there is no growth of B and lysis soon occurs.

(g) While there is good evidence that lysis is brought about by the attainment of a particular $[P]$ per B and not by a certain $[P]$ per ml., it is not clear at this time which of the ratios intracellular P/B, extracellular P/B or total P/B is the major conditioning factor for B lysis.

(h) Experimentally the maximal $[P]$'s of lysates made by mixing a constant initial $[B]$ with widely varying Po's fall within a relatively narrow range. This fact is explained by the large value of $d \log P/d\ t$ as compared to $d \log B/d\ t$. That is, the loci of points at which $\log P = 2.1 + \log B$ (maxima-lysis begins) on the curves of $\log P$ against t originating in various $[Po]$'s will lie at a nearly constant level above the abscissa. Because of this same relationship the maximal $[P]$'s of such a series will be in the reverse order of magnitude of the Po's, *i.e.*, the larger the Po the smaller will be the maximal $[P]$ attained during the reaction (*cf.* Fig. 16).

(*i*) The lytic destruction of B is logarithmic with time, in this respect being similar to most death rate processes. The value $-d \log B/d\ t$ for a particular initial $[B]$ is constant for various initial values of $[P]$. There is good evidence that cells need not be growing in order to undergo lysis.

(j) During B lysis a considerable percentage of the total maximal P formed is destroyed, the chief loss probably occurring in the intracellular fraction. The major portion (70 to 90 per cent) of the final P

present after the completion of bacteriophagy is set free during the brief phase of bacterial dissolution.

(k) When the entire process of bacteriophagy is completed the lysates are left with certain $[P]$'s determined by the foregone P-B reaction. The destruction of P during lysis is sufficiently regular to maintain the relationship established at the maximal $[P]$'s. Therefore the final $[P]$'s have the same points in common that were noted in "h" as applying to the maximal $[P]$'s. That is, they all are grouped within a narrow range of $[P]$ values, those having been made with high Po's being of lower titre than those made with low initial $[P]$'s.

(l) There is a significant difference in the temperature coefficients of P and B formation. Further, the temperature coefficients of P and B destruction during lysis differ in almost the same ratio. Consequently, while all experimental evidence postulates B growth as an essential conditioning factor for P formation, the temperature coefficient data suggest that the two processes are basically separate reactions. A similar interpretation holds in the case of B dissolution and P inactivation.

(m) The major events in the complete process of "bacteriophagy" are mathematically predictable. The $[B]$ at which lysis occurs under certain standard conditions for given values of Bo and Po may be calculated from the equation:

$$\text{Log } B = \frac{2.1 + n \log Bo - \log Po}{n - 1}$$

Substitution of this value for log B in the equation:

$$t = \frac{1}{g\,Be}\left[\log \frac{B\,(Be - Bo)}{Bo\,(Be - B)}\right]$$

gives satisfactory agreement with observed values for $t_{(\text{lysis})}$.

(n) The kinetic analysis of the P-B reaction predicts that the values of log Po plotted against $t_{(\text{lysis})}$ for a constant Bo will give a straight line. This plot is employed in a method for the quantitative estimation of P described in an earlier paper on the basis of experimental observation alone. Its use is made more rational by the facts given above.

The writers wish to acknowledge the assistance of Miss V. Toussaint in performing many of the experiments described in this paper.

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ELECTROMETRIC DETERMINATIONS OF THE DISSOCIATION OF GLYCOCOLL AND SIMPLE PEPTIDES[1]

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(Accepted for publication, September 19, 1930)

Because of their importance in biochemistry and in the theory of amphoteric electrolytes, the acid and basic dissociation constants of amino acids and polypeptides have been measured by numerous investigators. A summary of available data has been published by Kirk and Schmidt (1). The agreement among the results of the several investigators is not very good. This is due to numerous causes, chief among them being the ever present uncertainty of the liquid junction potentials in the potentiometric studies, the difficulty of preparing compounds of sufficient purity and the neglect of certain corrections due to ionic activity.

The first two named difficulties are mechanical in nature. A method of obviating the last was undertaken by Simms (2) in a series of titration experiments wherein he employed the Brönsted and LaMer (3) limiting equation to obtain the activity coefficients of the ionic species involved. He worked with glycocoll and studied its dissociation in the presence of NaCl and $BaCl_2$. The further electrometric titration of glycocoll and gelatin was conducted in the presence of certain antagonistic salt mixtures. The effect on the pH of the solution was complex.

The present paper reports the potentiometric determination of the apparent acidic and basic dissociation constants of glycocoll and several peptides in aqueous solution. Measurements were also made in the presence of KCl or of K_2SO_4 at equal ionic strength. Two methods, hydrolysis and titration, were employed. Hydrolysis afforded a basis of comparison of order of magnitude with the results

[1] This paper reports work done for a thesis submitted by Jesse P. Greenstein in partial fulfillment of the requirements for the degree of Ph.D. at Brown University.

of titration. This was desirable because uncertainties involved in determinations of hydrogen ion activities in a range far from the buffer region are clearly recognized. The hydrolysis method was suggested by the work of Denham (4) on inorganic salts. He pointed out that the potentiometric determination of the degree of hydrolysis constituted the most satisfactory method for this type of study, particularly in solutions of low hydrogen ion activity.

Winkelblech (5), on the other hand, employed the Bredig method of conductance to determine the degree of hydrolysis and studied several of the amino acids in this manner. But difficulties involved in the conductance method make it a rather unsatisfactory means for the study of the dissociation of these substances.

Materials and Apparatus

The following peptides were synthesized according to the general methods of Emil Fischer: glycylglycocoll (6), alanylglycocoll (7), leucylglycocoll (8), methylleucylglycocoll (9), valylglycocoll (10), phenylalanylglycocoll (11) and glycylglycylglycocoll (12). All compounds were carefully boiled with absolute alcohol until no trace of chloride remained. They were then crystallized twice from pure distilled water in some cases under addition of alcohol and were dried at 100°C. Most of the intermediates were likewise crystallized from the appropriate solvents or else fractionally distilled. Previous to use in the experiments the peptides were kept for several hours over phosphorus pentoxide. No specific tests for purity could be made inasmuch as the compounds exhibit a wide range of fusion with decomposition within the fusion interval.

The usual scheme of measuring hydrogen electrode potentials was employed with a Type K potentiometer, a carefully calibrated Eppley standard cell and a reflecting type of galvanometer with lamp and scale. A saturated calomel electrode was used. All materials for its preparation were carefully purified. Its constant, determined by checking against a tenth normal calomel electrode, was 0.2454.

Hydrochloric acid was prepared by distilling a high grade commercial product and taking the middle fraction. It was standardized against carefully dried, pure sodium carbonate using methyl orange as indicator. Sodium hydroxide was prepared according to the method of Cornog (13) and was standardized against the standard hydrochloric acid. The KCl and K_2SO_4 were from LaMotte and Kahlbaum stock respectively. The water was distilled from a Barnstead still and had a specific conductance of 1×10^{-6} mho. The salt bridge was the saturated KCl model recommended by Michaelis (14). This seems eminently satisfactory in comparison with other types in common use. The recent work of Guggenheim may be referred to in this connection (15).

All measurements were made in a thermostat at 25°C. ± 0.1°. Readings were taken until potentials checked to 0.2 mv. The value of pK_w at 25°C. was taken to be 13.895.

Hydrolysis Studies

The method of hydrolysis involves, essentially, the potentiometric determination of the hydrogen ion activity (a_{H^+} or pH) in equimolar mixtures of the peptide and either HCl or NaOH. Lùden (16) has given a theoretical treatment of this type of reaction but his formulations were made some time before the advent of the modern theories of solution.

The following method of formulating the reactions involved was suggested by the work of Fenwick and Gilman (17) on the dissociation of the salts of certain complex organic compounds.

It is assumed that the acid reacts with the compound to form the hydrochloride whereas the base reacts to form the sodium salt. Complete dissociation of the salt in either case is taken for granted.

Letting R represent the general molecular grouping of the ampholyte, the reactions occurring in acid solution may be given thus:

$$R^+ + Cl^- + HOH \rightleftharpoons ROH + Cl^- + H^+$$

$$\frac{a_{R^+} \times a_{HOH}}{a_{ROH} \times a_{H^+}} = \frac{k_b}{K_W} = \frac{a_{R^+}}{a_{ROH} \times a_{H^+}} = \frac{1}{K_a}$$

It is assumed that water possesses unit activity although Harned (18) in a series of calculations on the vapor pressure of water, showed that its activity at ordinary temperatures was slightly less than this value.

The degree of hydrolysis of the salt, represented by X, will be:

$$X = \frac{a_{H^+}}{\gamma_{H^+} \cdot C},$$

where C is the total concentration of ampholyte, reckoned on a molal basis, and γ_{H^+} is the activity coefficient of hydrogen ion concentration. Substituting in the mass action equation:

$$\frac{\lambda_{R^+}(1 - X)C}{\gamma_{ROH} \cdot XC\, a_{H^+}} = \frac{k_b}{K_W} = \frac{1}{K_a},$$

$$\frac{\gamma_{R^+}(\gamma_{H^+}C - a_{H^+})}{\gamma_{ROH}\, a^2_{H^+}} = \frac{1}{K_a}$$

Converting the expression into logarithmic form,

$$\log [\gamma_{H^+}C - a_{H^+}] + \log \gamma_R - \log \gamma_{ROH} + 2pH = pK_a$$

The values of the mean activity coefficient of hydrogen ion, γ_{H^+}, were taken from Schatchard's data (19) on solutions of HCl. It is assumed that, within the experimental error, the activity coefficient of hydrogen ion in the presence of ampholyte is approximately the same as in pure acid solution.

In order to determine the activity coefficient of the ampholyte ion, the ionic strength of the solution must be taken into account. Debye and Hückel (20), employing the assumption of complete dissociation of strong electrolytes, elaborated a theory which accounts for the deviations from the ideal state by taking into consideration the electrical forces among the ions. Their mathematical treatment was further simplified by Brönsted and LaMer (21) who found that the activity coefficient was related to the ionic strength, μ, of the solution by the equation: $-\log \gamma = Az^2\sqrt{\mu}$, where A is a coefficient depending upon the dielectric constant of the medium and the absolute temperature and z refers to the valence. This equation is employed in the following formulation. The coefficient, A, at 25°C., is very nearly 0.5, so that, $\log \gamma_{R^+} = -0.5z^2\sqrt{\mu} = -0.5\sqrt{C}$, and assuming that the activity coefficient of the undissociated portion of a weak electrolyte is very nearly unity, the working equation is obtained:

$$\log [\gamma_{H^+}\, C - a_{H^+}] - 0.5\sqrt{C} + 2pH - pK_a$$

A similar equation, based on reactions in alkaline solutions, is:

$$\log [\gamma_{OH^-}C - a_{OH^-}] - 0.5\sqrt{C} + 2pOH = pK_b$$

Values of γ_{OH^-} are the mean values taken from Harned's data on NaOH solutions.

The following compounds were studied by the hydrolysis method: glycocoll, glycylglycocoll, alanylglycocoll, leucylglycocoll, methylleucylglycocoll, valylglycocoll, phenylalanylglycocoll, and glycylglycylglycocoll.

The experimental procedure consisted in making up a normal solution of either HCl or NaOH in a volumetric flask and adding an equivalent normal weight of ampholyte. In some cases the initial solution was 0.1N, in others, particularly among the less soluble variety, the normality was lower. 50 cc. of the original solution were removed, transferred to another flask (100 cc.) and made up to the mark with distilled water. Several successive dilutions were made in this manner whereby the equivalence of peptide to reagent was kept constant and the concentration halved at each successive dilution. The pH of each dilution was determined at 25°C. and the dissociation constants calculated.

The potentials obtained in the case of phenylalanylglycocoll were erratic and considerable time elapsed before equilibrium was reached. Believing this to be an effect of the platinum black, we employed a quinhydrone electrode arranged according to Biilmann (22) in the hope of attaining equilibria more rapidly. This electrode yielded no better results, however, and it was discarded. Abderhalden and Suzuki (23) have shown that peptides containing aromatic radicals are rapidly split at the imide linkage in the presence of dilute acid and alkali even at ordinary temperatures.

The measurements by the hydrolysis method and the computed constants, pK_a and pK_b, are presented in Tables I and II. The results for glycocoll are less consistent than those for the peptides. This is probably due to impurity of the glycocoll.

A consideration of the deviations of the dissociation constants with variation in ionic strength, indicates in general a decided shift toward higher values of K_a and lower values of K_b as the ionic concentration is increased. These constants should give, if the formulations were adequate, a uniform value in the case of each compound. The mass action law has been stated in the requisite terms of activities; the failure to yield constants of more uniform character merits a critical analysis of the method of formulation.

It has been assumed that the activity coefficient of the ampholytic ion R, may be expressed in terms of the limiting law:

$$-\log \gamma_R = 0.5\, z^2 \sqrt{\mu}$$

whereby the activity coefficient is solely a function of the valence and the ionic strength. Such a relationship will hold only in extremely dilute solutions ($\mu < .01$) where the ionic diameters may be neglected. In the case of long ions, the fundamental equations of Debye and Hückel must be modified although as yet no theoretical treatment of the spatial distribution of charges has been attempted.

TABLE I

Measurements by the Hydrolysis Method in Acid Solution

C	$0.5\sqrt{C}$	Glycocoll		Glycylglycocoll		Valylglycocoll		Alanylglycocoll	
		pH	pK_a	pH	pK_a	pH	pK_a	pH	pK_a
0.100	0.157	1.778	2.238	2.116	2.958	.....	.....	2.159	3.048
0.050	0.110	1.949	2.293	2.277	3.023	.....	.....	2.328	3.129
0.025	0.080	2.138	(2.372)	2.450	3.087	2.478	3.148	2.477	3.145
0.012	0.055	2.304	2.331	2.637	3.158	2.656	3.201	2.637	3.158
0.006	0.0385	2.490	2.322	2.785	3.134	2.810	3.193	2.795	3.154
0.003	0.0275	2.702	2.331	2.974	3.175	3.021	3.292	2.967	3.161

C	$0.5\sqrt{C}$	Leucylglycocoll		Methylleucylglycocoll		Phenylalanylglycocoll		Glycylglycylglycocoll	
		pH	pK_a	pH	pK_a	pH	pK_a	pH	pK_a
0.100	0.157	2.165	3.060	.....	.....	.....	.....	2.191	3.114
0.050	0.110	2.324	3.121	2.465	3.398	.....	.....	2.355	3.186
0.025	0.080	2.467	3.123	2.587	3.384	2.042	2.118	2.524	3.249
0.012	0.055	2.632	3.148	2.715	3.334	2.204	2.034	2.666	3.226
0.006	0.0385	2.796	3.156	2.869	3.332	2.416	2.048	2.832	3.248
0.003	0.0275	2.959	3.146	3.022	3.294	2.648	2.046	3.003	3.256

TABLE II

Measurements by the Hydrolysis Method in Basic Solution

C	$0.5\sqrt{C}$	Glycocoll		Glycylglycocoll		Valylglycocoll		Alanylglycocoll	
		pOH	pK_b	pOH	pK_b	pOH	pK_b	pOH	pK_b
0.050	0.110	2.879	4.293	3.687	5.920	.....	.....	3.870	6.287
0.025	0.080	3.019	4.309	3.824	5.933	4.048	6.383	3.933	6.157
0.012	0.055	3.159	4.296	3.987	5.976	4.175	6.355	4.071	6.147
0.006	0.0385	3.312	4.317	4.166	6.056	4.312	6.348	4.193	6.111
0.003	0.0275	3.432	4.251	4.301	6.036	4.479	6.393	4.323	6.081

C	$0.5\sqrt{C}$	Leucylglycocoll		Methylleucylglycocoll		Phenylalanylglycocoll		Glycylglycylglycocoll	
		pOH	pK_b	pOH	pK_b	pOH	pK_b	pOH	pK_b
0.050	0.110	3.907	6.361	3.811	6.169	.....	.....	3.912	6.371
0.025	0.080	3.975	6.235	3.895	6.075	3.202	4.680	4.026	6.339
0.012	0.055	4.100	6.205	4.019	6.043	3.542	5.982	4.165	6.335
0.006	0.0385	4.251	6.227	4.175	6.075	3.894	5.505	4.313	6.351
0.003	0.0275	4.405	6.245	4.320	6.075	4.215	5.865	4.461	6.357

Simms has suggested an empirical formula to apply to certain large organic ions. His equations however are confined to those concentrations where μ varies from 0.01 to 0.1.

In the application of the fundamental equation, it has also been assumed that the peptide ion is univalent. Such a conception, in view of the several reactions described in the Introduction, seems somewhat doubtful. While the monovalent hydrochlorides of the peptides may be easily isolated, it is uncertain that such combinations are strictly stoichiometric. However, the question of extra valencies within the peptide molecule is so far from being settled that it need not be further considered here.

This leaves the problem of the ionic diameters to be considered. Probably this is where the present calculations are most in error. The simple limiting equation holds in concentrations where $\gamma < 0.01$. Above this limit, the activity coefficient of an ion must be a function of some value characteristic of all the ionic diameters in the solution. Where this factor is considered, the activity coefficient is defined by Hückel (24) as follows:

$$-\log \gamma = \frac{0.5\, z^2 \sqrt{\mu}}{1 + 3.3 \times 10^7\, r \sqrt{\mu}} + B(2\mu)$$

Here r may be interpreted as the average effective diameter of all the ions. Data relating to this variable are meager in the case of inorganic ions and are practically non-existent in the case of organic ions. The second term on the right hand side of the above equation represents the effect of the solute concentration on the dielectric constant. The zwitter ion with its dual charge and permanent moment might be expected to influence the dielectric constant considerably, and the experiments of Blüh (25) indicate that its value increases progressively with the concentration. Beyond the fact that at higher concentrations the dielectric constant of the solution is no longer the same as that of the pure solvent, little hope of arriving at the nature of the solute from capacity measurements can at present be held. It may be that the zwitter ion is an association complex in the sense of Bjerrum (26). Recent very accurate measurements (27) indicate that aqueous solutions of strong electrolytes slightly depress the dielectric constant of the solvent.

In view of the paucity of our knowledge concerning this problem, it seems best to leave the dissociation constants in their present form, referring to them as "apparent" constants since they are functions of several variables.

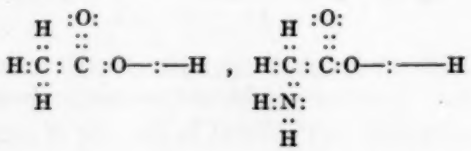
Turning our attention to the relative order of magnitude of the constants of the various compounds, we see a partial proof of the conceptions of Bjerrum and Adams.

Considering acid constants alone, their relative order of magnitude may be represented as follows:

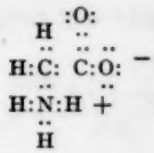
Phenylalanylgycocoll > glycocoll > glycylglycocoll > (alanylglycocoll, leucylglycocoll, valylglycocoll) > glycylglycylglycocoll > methylleucylglycocoll.

Bjerrum and Adams postulated a high degree of acidity for the glycocoll molecule in consequence of the presence of the highly electronegative group. The latter will further the dissociation of the hydrogen ion from the carboxyl group, such influence decreasing as the amino group is removed further from the carboxyl.

Representing the electronic structure of acetic acid and glycocoll thus:



it is apparent why glycocoll is a far stronger acid than acetic (some 100 times) and yet is neutral in pure water solution. The highly electronegative amino group will effect a deformation in the electronic nucleus, causing a shift in the position of the electron pairs about the carbon and oxygen atoms of the carboxyl group. This shift is toward the amino group. In the case of the oxygen-hydrogen linkage of the carboxyl group, the electron pair is drawn closer to the oxygen and farther away from the hydrogen. This amounts to a decrease in the strength of the bond, and an increase in the degree of dissociation of the acid. The proton thus set free combines immediately with the two unshared electrons of the nitrogen, forming the dipolar molecule, or zwitter ion.



It is apparent therefore that the farther away the amino group is from the carboxyl (the greater the number of carbon atoms intervening), the smaller will be the deformation of the electronic nucleus and the slighter the dissociation. Hence the observed decrease in the relative magnitude of the dissociation constants.

Two apparently significant exceptions occur to this rule, namely, in the case of phenylalanylglycocoll and of methylleucylglycocoll. In the former compound it is to be expected that the very strongly electronegative character of the phenyl group would be sufficient to make the compound a stronger acid than glycocoll. In the latter, introducing a methyl radical into the amino group decreases its electronegativity. This lowering of hydrogen-accepting capacity is apparently decreased as the dilution increases, and the compound forms an apparent exception to the rule of increased value of acid dissociation constant with increasing hydrogen ion activity.

In alkaline solution, the basic dissociation constants in general show a decided increase in value as the ionic strength decreases.

As a result of these particular studies, the generalization may be advanced that the dissociation constants are functions of the hydrogen ion activity and the ionic strength.

Titration Studies

The following methods of formulating the reactions involved in titration measurements were suggested in part by the work of Simms and the discussions of Kolthoff (28), but several modifications have been introduced. If we consider the reaction in acid solution, we may formulate the equilibrium thus:

$$^{+}X^{-} + H^{+} = H^{+}X$$

where $^{+}X^{-}$ refers to the zwitter ion, $NH_3^{+}RCOO^{-}$. In consequence of the small ionic product of water, the concentration of ampholytic

anions in the presence of acid will be negligible. In mass action form, the equation is:

$$\frac{H^+ \times {}^+X^-}{H^+X} = K'_a$$

If α represents the apparent "degree of dissociation" or the mol fraction of ampholytic cations ($\alpha = \frac{H^+X}{C}$, where C is the total concentration of ampholyte) then the mass action equation may be written thus:

$$K'_a = a_{H^+} \frac{(1 - \alpha)}{\alpha}$$

and in logarithmic form:

$$pK'_a = pH - \log \frac{(1 - \alpha)}{\alpha}$$

For electroneutrality in the solution the sum of the concentrations of cations must equal that of the anions or:

$$H^+ + H^+X = OH^- + Cl^-$$

and, assuming complete dissociation of HCl and negligible concentration of hydroxyl ions,

$$H^+ + H^+X = A$$

where A is the total concentration of acid.

It follows that:

$$H^+ + \alpha C = A \text{ and } \alpha = \frac{A - H^+}{C}$$

but, inasmuch as α is to be expressed as concentration, the H^+ values, observed as activities, must be converted to concentrations by means of the equations:

$$H^+ = \frac{a_{H^+}}{\gamma_{H^+}} \text{ and } \log \gamma_{H^+} = -0.5\sqrt{\mu}$$

In basic solution, the reactions may be formulated thus:

$$^{+}X^{-} + OH^{-} = XOH^{-} \quad \text{and} \quad \frac{^{+}X^{-} \times OH^{-}}{XOH^{-}} = K'_b$$

Representing α as the mol fraction of ampholytic anion, $\alpha = \frac{XOH^{-}}{C}$,

$$K'_b = a_{OH^-} \frac{(1 - \alpha)}{\alpha} \quad \text{and} \quad pK'_b = pOH - \log \frac{(1 - \alpha)}{\alpha}$$

For electroneutrality:

$$H^{+} + Na^{+} = OH^{-} + XOH^{-}$$

and assuming the sodium hydroxide, B, as completely dissociated:

$$H^{+} + B = OH^{-} + \alpha C$$

whence

$$\alpha = \frac{B + H^{+} - OH^{-}}{C}$$

Hydroxyl ion concentrations were calculated from the concentrations of hydrogen ions by means of the ionic product of water at 25°, $K_w = 1.27 \times 10^{-14}$.

The dissociation constants so calculated are of a hybrid or "apparent" character, inasmuch as the expression $\log \frac{1 - \alpha}{\alpha}$ is expressed as concentration while pH is the negative logarithm of the hydrogen ion activity.

In order to obtain the "true" dissociation constants which shall be independent of the ionic strength of the solution, it is necessary to have an accurate knowledge of the activity coefficients of the ampholyte ions. Any attempt to derive a value for these coefficients on the basis of the Debye-Hückel theory involves certain *a priori* assumptions which have been considered in the previous section on hydrolysis.

The experimental technique consisted in adding acid or base to a constant quantity of ampholyte alone in solution, and in the presence of salt. A 0.002 N solution of either HCl or NaOH was made up in a 200 cc. volumetric flask. To

this was added a weighed amount of ampholyte so that its concentration would be 0.02 M. 25 cc. samples were placed in 50 cc. volumetric flasks with the desired amounts of 0.1 N HCl or NaOH, and with 5 cc. of 0.5 M KCl or 0.166 M K_2SO_4, or with no salt. The aliquots were then made up to the mark with distilled water. The ionic strength, then, of each of the salts was the same (0.05μ). The final concentration of total ampholyte in each sample was 0.01 M.

Potentiometric data were obtained on the following compounds: glycocoll, glycylglycocoll, alanylglycocoll, leucylglycocoll, methylleucylglycocoll, and glycylglycylglycocoll. The dissociation constants of each ampholyte were calculated by means of the above formulae. The results are reported in Tables III–VIII. The lack of a sufficient supply of glycylglycylglycocoll was the cause of the paucity of data on this compound.

TABLE III

Electrometric Titration of Glycocoll (0.01 M)
With HCl

A	HCl without salt		HCl with KCl = 0.05μ		HCl with K_2SO_4 = 0.05μ	
	pH	pK'_a	pH	pK'_a	pH	pK'_a
0.001	3.419	2.232	3.495	2.292	3.614	2.484
0.002	3.123	2.262	3.182	2.295	3.279	2.461
0.003	2.973	2.335	2.979	2.271	3.093	2.480
0.004	2.818	2.311	2.849	2.289	2.962	2.506
0.005	2.707	2.314	2.725	2.257	2.852	2.516
0.006	2.612	2.311	2.629	2.244	2.744	2.502
0.007	2.511	2.264	2.539	2.209	2.668	2.526
0.008	2.450	2.287	2.467	2.200	2.597	2.540

Titration with NaOH

B	NaOH without salt		NaOH with KCl = 0.05μ		NaOH with K_2SO_4 = 0.05μ	
	pH	pK'_b	pH	pK'_b	pH	pK'_b
0.001	8.715	4.226	8.656	4.285	8.697	4.244
0.002	9.116	4.174	9.033	4.257	9.059	4.231
0.003	9.319	4.204	9.262	4.271	9.267	4.256
0.004	9.526	4.185	9.456	4.257	9.462	4.251
0.005	9.678	4.208	9.621	4.267	9.631	4.257
0.006	9.842	4.215	9.773	4.289	9.792	4.268
0.007	10.003	4.238	9.942	4.305	9.974	4.271
0.008	10.184	4.268	10.133	4.330	10.135	4.328

TABLE IV

Electrometric Titration of Glycylglycocoll (0.01 M)
With HCl

A	HCl without salt		HCl with KCl = 0.05μ		HCl with K_2SO_4 = 0.05μ	
	pH	pK'_a	pH	pK'_a	pH	pK'_a
0.001	4.110	3.115	4.145	3.146	4.128	3.129
0.002	3.790	3.140	3.790	3.128	3.829	3.173
0.003	3.541	3.109	3.578	3.137	3.622	3.188
0.004	3.362	3.101	3.392	3.119	3.458	3.199
0.005	3.218	3.104	3.248	3.119	3.309	3.197
0.006	3.077	3.092	3.108	3.100	3.179	3.203
0.007	2.944	3.075	2.969	3.084	3.049	3.194
0.008	2.810	3.041	2.849	3.049	2.945	3.216

Titration with NaOH

B	NaOH without salt		NaOH with KCl = 0.05μ		NaOH with K_2SO_4 = 0.05μ	
	pH	pK'_b	pH	pK'_b	pH	pK'_b
0.001	7.304	5.637	7.047	(5.894)	7.267	5.674
0.002	7.676	5.617	7.590	5.703	7.607	5.686
0.003	7.854	5.673	7.792	5.735	7.818	5.709
0.004	8.059	5.660	7.993	5.726	8.003	5.716
0.005	8.225	5.670	8.162	5.733	8.169	5.726
0.006	8.387	5.684	8.338	5.733	8.356	5.715
0.007	8.594	5.669	8.533	5.730	8.539	5.724
0.008	8.803	5.694	8.758	5.739	8.769	5.728

TABLE V

Electrometric Titration of Alanylglycocoll (0.01 M)
With HCl

A	HCl without salt		HCl with KCl = 0.05μ		HCl with K_2SO_4 = 0.05μ	
	pH	pK'_a	pH	pK'_a	pH	pK'_a
0.001	.....	.....	4.164	3.169	4.206	3.211
0.002	3.773	3.123	3.813	3.155	3.857	3.205
0.003	3.571	3.143	3.610	3.176	3.649	3.219
0.004	3.399	3.143	3.423	3.156	3.473	3.217
0.005	3.248	3.141	3.275	3.154	3.326	3.220
0.006	3.106	3.132	3.126	3.129	3.204	3.237
0.007	2.967	3.110	2.976	3.084	3.064	3.217
0.008	2.839	3.085	2.866	3.058	2.964	3.246

Titration with NaOH

B	NaOH without salt		NaOH with KCl = 0.05μ		NaOH with K_2SO_4 = 0.05μ	
	pH	pK'_b	pH	pK'_b	pH	pK'_b
0.001	7.331	5.610	7.260	5.681	7.277	5.664
0.002	7.688	5.605	7.619	5.674	7.629	5.664
0.003	7.886	5.641	7.839	5.688	7.844	5.683
0.004	8.071	5.648	8.011	5.708	8.033	5.686
0.005	8.248	5.647	8.186	5.709	8.211	5.684
0.006	8.423	5.648	8.377	5.694	8.380	5.691
0.007	8.631	5.632	8.570	5.693	8.588	5.675
0.008	8.837	5.660	8.778	5.719	8.792	5.705

TABLE VI

Electrometric Titration of Leucylglycocoll (0.01 M)
With HCl

A	HCl without salt		HCl with KCl = 0.05μ		HCl with K_2SO_4 = 0.05μ	
	pH	pK'_a	pH	pK'_a	pH	pK'_a
0.001	4.152	3.163	4.176	3.181	4.194	3.199
0.002	3.798	3.151	3.808	3.151	3.849	3.197
0.003	3.577	3.149	3.609	3.173	3.649	3.219
0.004	3.392	3.136	3.434	3.171	3.477	3.221
0.005	3.252	3.147	3.275	3.154	3.328	3.222
0.006	3.103	3.127	3.132	3.139	3.191	3.219
0.007	2.978	3.127	2.988	3.103	3.072	3.230
0.008	2.840	3.090	2.874	3.094	2.969	3.255

Titration with NaOH

B	NaOH without salt		NaOH with KCl = 0.05μ		NaOH with K_2SO_4 = 0.05μ	
	pH	pK'_b	pH	pK'_b	pH	pK'_b
0.001	7.209	5.733	7.154	5.787	7.164	5.777
0.002	7.577	5.716	7.499	5.794	7.506	5.787
0.003	7.768	5.759	7.709	5.818	7.712	5.815
0.004	7.968	5.751	7.905	5.814	7.910	5.809
0.005	8.133	5.762	8.081	5.814	8.071	5.824
0.006	8.321	5.750	8.253	5.818	8.260	5.811
0.007	8.511	5.752	8.448	5.815	8.451	5.812
0.008	8.725	5.772	8.653	5.844	8.663	5.834

TABLE VII

Electrometric Titration of Methylleucylglycocoll (0.01 M)
With HCl

A	HCl without salt		HCl with KCl = 0.05μ		HCl with K_2SO_4 = 0.05μ	
	pH	pK'_a	pH	pK'_a	pH	pK'_a
0.001	4.216	3.232	4.226	3.237	4.184	3.189
0.002	3.846	3.205	3.890	3.243	3.913	3.269
0.003	3.631	3.212	3.665	3.237	3.698	3.275
0.004	3.453	3.208	3.490	3.236	3.521	3.273
0.005	3.304	3.212	3.331	3.225	3.377	3.281
0.006	3.154	3.196	3.184	3.210	3.233	3.275
0.007	3.011	3.175	3.040	3.182	3.109	3.283
0.008	2.879	3.155	2.912	3.162	2.996	3.298

Titration with NaOH

B	NaOH without salt		NaOH with KCl = 0.05μ		NaOH with K_2SO_4 = 0.05μ	
	pH	pK'_b	pH	pK'_b	pH	pK'_b
0.001	7.340	5.601	7.289	5.652	.....	.....
0.002	7.680	5.613	7.636	5.657	7.648	5.645
0.003	7.891	5.636	7.849	5.678	7.851	5.676
0.004	8.089	5.630	8.032	5.687	8.037	5.682
0.005	8.257	5.638	8.208	5.687	8.213	5.682
0.006	8.434	5.637	8.396	5.675	8.387	5.684
0.007	8.629	5.634	8.575	5.688	8.587	5.676
0.008	8.840	5.657	8.797	5.700	8.800	5.697

TABLE VIII

Electrometric Titration of Glycylglycylglycocoll (0.01 M)
With HCl

A	HCl without salt		HCl with KCl = 0.05μ	
	pH	pK'_a	pH	pK'_a
0.001	4.209	3.225	4.236	3.247
0.003	3.648	3.231	3.671	3.246
0.005	3.318	3.229	3.353	3.252
0.007	3.032	3.204	3.052	3.199

Titration with NaOH

B	NaOH without salt		NaOH with KCl = 0.05μ	
	pH	pK'_b	pH	pK'_b
0.001	7.003	(5.938)	7.032	5.909
0.003	7.656	5.871	7.615	5.912
0.005	8.035	5.860	7.978	5.917
0.007	8.390	5.873	8.372	(5.891)

DISCUSSION

On an examination of the data, the following facts are brought to light. The first is that in general the dissociation constants are a function of the hydrogen ion activity. They show definite trends: the values of K_a and K_b of each compound increasing with increasing hydrogen ion activity in solution without the presence of salt and in the presence of KCl; while K_a in the presence of K_2SO_4 decreases with hydrogen ion activity whereas K_b in the presence of K_2SO_4 increases. At equal ionic strengths the acid constants in the presence of KCl are greater than in the presence of K_2SO_4, the reverse holding true of the basic constants in alkaline solution.

The following chart indicates the trend of the apparent dissociation constants in each titration series with H^+ ion activity and ionic strength: the arrows pointing upwards indicate increasing values, those downward, decreasing values.

Without salt	$K'_a \uparrow$	$a_{H^+} \uparrow$	$\mu \uparrow$
	$\vee$	$\vee$	$\wedge$
With KCl	$K'_a \uparrow$	$a_{H^+} \uparrow$	$\mu \uparrow$
	$\vee$	$\vee$	$\|\|$
With K_2SO_4	$K'_a \downarrow$	$a_{H^+} \uparrow$	$\mu \uparrow$

Without salt	$K'_b \uparrow$	$a_{H^+} \uparrow$	$\mu \downarrow$
	$\vee$	$\wedge$	$\wedge$
With K_2SO_4	$K'_b \uparrow$	$a_{H^+} \uparrow$	$\mu \downarrow$
	$\vee$	$\wedge$	$\|\|$
With KCl	$K'_b \uparrow$	$a_{H^+} \uparrow$	$\mu \downarrow$

A satisfactory explanation of the deviations of these constants is difficult to give on purely theoretical grounds. It is known that the apparent dissociation constant of a weak electrolyte is markedly dependent upon the ionic strength of the solution, and in the case of the ampholyte should decrease in absolute value on the addition of other electrolytes.

Stated in terms of the simplified Debye-Hückel theory:

$$-\log \gamma = 0.5\, z^2 \sqrt{\mu},$$

if the equilibrium of the ampholyte be represented as above,

$$K'_a = a_{H^+} \frac{(1 - \alpha)}{\alpha}$$

where K'_a is the apparent dissociation constant, then the true dissociation constant based on activities will be $K_a = K'_a \frac{1}{\gamma}$, assuming that the activity coefficient of the undissociated ampholyte is equal to unity.

Therefore

$$\log K'_a = \log K_a - 0.5 z^2 \sqrt{\mu}$$

Inasmuch as K_a is a constant, the apparent dissociation constant should decrease as the ionic strength increases. The same relation should hold for K'_b.

On the contrary, in acid solution in each titration series with the exception of those involving K_2SO_4, we observe an increase of the apparent constant with increasing ionic strength. Furthermore, according to the principle of ionic strength, the constants in the presence of the salts should be equal at equal ionic strengths. It must be remembered, however, that this law is of limited validity, holding only in such very dilute solutions that specific ion effects are negligible. In the present studies, the difference in the specific effects of the sulfate and chloride ions is clearly evidenced. The work of Harned (29) on the activity coefficients of sulfuric acid in the presence of sulfate ion is illuminating in this regard. He found a decreased activity of the acid in the presence of this ion as the latter's concentration increased. It seems probable that the decrease is due to the formation of HSO_4 ions. Kraus (30), listing the activity coefficients of K_2SO_4 solutions, indicated the very low activity coefficient of this salt as compared with that of KCl. The latter, for example, at 0.01 M has a value of 0.922, the former at the same concentration a value of 0.687. Sulfuric acid yields a coefficient of 0.617. The effect of the sulfate ion will be to lower the ionic activity of the medium; increase in ionic strength will then so favor the formation of ampholyte ions that their excess will outweigh the effect of reduced hydrogen ion activity on the magnitude of the apparent constant. This is indicated by a comparison of α values and H^+ ion activities in acid-ampholyte, KCl-ampholyte, and K_2SO_4-ampholyte solutions:

$$\alpha_{K_2SO_4} > \alpha_{\text{no salt}} > \alpha_{KCl}$$

$$a_{H^+\text{no salt}} > a_{H^+KCl} > a_{H^+\ K_2SO_4}$$

It may be that the lowering of the degree of dissociation in the presence of KCl is due to a common ion effect. The apparent disagreement of K'_a with the Debye-Hückel equation confirms the work of Simms on glycocoll.

The agreement of the equation with theory in the case of the apparent basic constants is substantially good. Here the constants in general decrease as the ionic strength is increased.

It would be unwise to speculate further on specific ion effects in view of the slight amount of data now available in the literature. The present paper is simply a contribution to the study of ampholytes in salt solutions, and a more nearly complete elucidation must await further theoretical advances.

SUMMARY

1. The apparent acid and basic dissociation constants were determined potentiometrically by the methods of hydrolysis and of titration for the following ampholytes: Glycocoll, glycylglycocoll, alanylglycocoll, valylglycocoll, leucylglycocoll, methylleucylglycocoll, phenylalanylglycocoll and glycylglycylglycocoll. The constants were also determined in the presence of KCl and of K_2SO_4 at equal ionic strength.

2. In general, the relative order of magnitude of the constants decreased as the number of carbon atoms between amino and carboxyl groups increased. An explanation of this is offered on the basis of theories of electronic structure.

3. The application of the modern concepts of solutions to the case of the ampholytic ions is discussed. The inadequacy of the present theories is pointed out.

4. The constants were found, in general, to be functions of the hydrogen ion activity and the ionic strength of the solutions. Apparent contradictions to the Debye-Hückel theory are pointed out and partially explained on the basis of specific ion effects.

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THE KINETICS OF PENETRATION

III. Equations for the Exchange of Ions

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(Accepted for publication, August 1, 1930)

The assumption is frequently made that an exchange of ions occurs in living cells. Since thermodynamics affords no test of this assumption the problem must be attacked kinetically and a mathematical treatment is therefore necessary. The brief outline given here is a preliminary attempt in this direction.

In the subsequent treatment we shall for convenience assume that the activity coefficients equal unity, that the electrolytes are monovalent, that when the concentrations of electrolytes are not the same within and without the osmotic pressures are rendered equal by the presence of non-electrolytes,[1] and that conditions are such that if a non-electrolyte penetrates, the time curve is of the first order. It is also convenient to assume that the solutions are not buffered so that when H^+ enters it appears as such inside.[2]

Supposing an exchange of cations, the protoplasm being impermeable to anions, it is evident that, other things being equal, the rate of exchange will be proportional to the activities (here equivalent to concentrations). For example, if we have chiefly H^+ outside and K^+ inside the rate at which H^+ passes inward in exchange for K^+ will be proportional to H_oK_i, where H_o is the external concentration of H^+ and K_i is the internal concentration of K^+. The outward passage of H^+ (in exchange for K^+) will be proportional to H_iK_o, so that the net rate will be proportional to the difference between the two, or to $H_oK_i - H_iK_o$.

[1] In case a divalent cation is exchanged for 2 monovalent cations there will be a change in osmotic pressure and a corresponding movement of water.

[2] In case the solutions are buffered we can put H_i equal to the titratable acidity in place of the concentration of H^+.

Let us now consider conditions at equilibrium.[3] Since all the anions are indiffusible we shall have a special kind of Donnan equilibrium with the relation (quite the opposite of that where the anions are diffusible)

$$\frac{A_o}{A_i} = \frac{C_o}{C_i}$$

where A is the total concentration of (monovalent) anions and C is the total concentration of (monovalent) cations. If the anions consist of several species A_1, A_2, etc., we shall have

$$\frac{A_{1o}}{A_{1i}} = \frac{A_{2o}}{A_{2i}} = \frac{A_o}{A_i}$$

and in the same way

$$\frac{C_{1o}}{C_{1i}} = \frac{C_{2o}}{C_{2i}} = \frac{C_o}{C_i} = \frac{A_o}{A_i}$$

Presumably the rate of exchange, other things being equal, will depend on the slower ion. Assuming that K^+ moves more slowly through the protoplasm[4] than H^+ we may say that since H^+ cannot pass through the protoplasm unless K^+ passes through at the same time in the opposite direction we need only consider the rate of movement of K^+. We may define the permeability to K^+, which we may call P_K, as the amount of K^+ passing through the protoplasm into the vacuole in unit time under standard conditions, *i.e.*, when the protoplasm has unit surface and when the value of $H_oK_i - H_iK_o$ equals unity.[5]

We may then put[6] for the net amounts passing through

$$-\frac{dK_i}{dt} = \frac{dH_i}{dt} = P_K\,[(H_o)\,(K_i) - (H_i)\,(K_o)]$$

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[3] *Cf.* Netter, H., *Arch. ges. Physiol.*, 1928, **220**, 107.

[4] The movement through the entire protoplasm (consisting of both aqueous and non-aqueous layers) is here referred to: it may be complicated by chemical combination, adsorption, etc.

[5] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

[6] Here

$$-\frac{dK_i}{dt} = \frac{dK_o}{dt}$$

and as the solutions are not buffered

$$\frac{dH_i}{dt} = -\frac{dH_o}{dt}$$

If the amount of K_i at the beginning be called K_{ib} we may say that (since K^+ must go out when H^+ comes in) K_i must equal K_{ib} less the net amount of H^+ which has entered, which is $H_i - H_{ib}$ (where H_{ib} denotes the amount of H_i at the beginning). Hence we have

$$K_i = K_{ib} - (H_i - H_{ib})$$

Substituting this value we have[7]

$$\frac{dH_i}{dt} = P_K [(H_o)(K_{ib}) + (H_o)(H_{ib}) - (H_o)(H_i) - (H_i)(K_o)]$$

If the external volume is sufficiently large H_o may be regarded as constant and if the concentration of K^+ is sufficiently small we may put $K_o = 0$. We may then write

$$K_{ib} + H_{ib} = C \text{ (which is a constant)}$$

and we have

$$\frac{dH_i}{dt} = P_K H_o (C - H_i)$$

---

[7] For example, if the internal and external volumes are equal and we put $H_{ob} = 95$, $H_{ib} = 10$, $K_{ib} = 37.5$ and $K_{ob} = 0$, then when 25 H^+ has gone in and 25 K^+ has come out we have equilibrium and

$$\frac{A_o}{A_i} = \frac{95}{47.5} = 2; \text{ also } \frac{H_o}{H_i} = \frac{70}{35} = 2, \text{ and } \frac{K_o}{K_i} = \frac{25}{12.5} = 2$$

Hence we have at equilibrium

$$\frac{dH_i}{dt} = P_K [(H_o)(K_{ib}) + (H_o)(H_{ib}) - (H_o)(H_i) - (H_i)(K_o)]$$

$$= P_K (70)(37.5) + (70)(10) - (70)(35) - (35)(25)$$

$$= P_K (0)$$

$$= 0$$

In dealing with equilibrium P_K plays no part but it would of course determine the course of the time curve since it is the apparent velocity constant of the process.

Putting $P_K H_o = V$ we have

$$V = \frac{1}{t} \ln_e \frac{C}{C - H_i}$$

It is evident that practically all of the K will leave the cell before equilibrium is reached.[8]

If the concentration of K in the external solution is not negligible but the external solution is relatively large the amount of K^+ coming out will make practically no change in the external concentration so that we may regard K_o and H_o as constant. We then[9] have (starting with the equation already given)

$$\frac{dH_i}{dt} = P_K [(H_o)(K_{ib}) + (H_o)(H_{ib}) - (H_o)(H_i) - (H_i)(K_o)]$$

---

[8] If, for example, $H_{ob} = 100$, $H_{ib} = 0$, $K_{ib} = 10$, and H_o is constant we should have at equilibrium $H_{ib} = 10$, and in order to arrive at this all of the K^+ inside would have to be replaced by H^+. Theoretically, however, the entrance of H^+ would diminish H_o slightly so that H_{ib} at equilibrium would be slightly less than ten and this slight margin would allow a very small quantity of K^+ to persist inside.

The values at equilibrium would be determined by the values of the anions A_o and A_i (see p. 278). In this case $A_o = 100$ and $A_i = 10$ so that at equilibrium we should have

$$\frac{A_o}{A_i} = \frac{100}{10} = 10 \text{ and (approximately) } \frac{H_o}{H_i} = \frac{100}{10} = 10$$

The value of $\frac{K_o}{K_i}$ must also equal 10 but since K_o and K_i are exceedingly small they may be neglected and we may put (approximately)

$$\frac{dH_i}{dt} = P_K H_o (K_{ib} + H_{ib} - H_i)$$

$$= P_K H_o (10 + 0 - 10)$$

$$= P_K H_o (0)$$

$$= 0$$

[9] To test this numerically let us put $H_{ob} = 50$, $K_{ob} = 50$, $K_{ib} = 10$, and $H_{ib} = 0$. At equilibrium we have

$$\frac{A_o}{A_i} = \frac{50 + 50}{10} = 10 \text{ and } \frac{H_{ob}}{H_{ib}} = \frac{50}{5} = 10; \text{ also } \frac{K_{ob}}{K_{ib}} = \frac{50}{5} = 10$$

$$= P_K [(H_o)(C) - (H_o)(H_i) - (H_i)(K_o)]$$

$$= P_K H_o \left[C - H_i - \frac{(H_i)(K_o)}{H_o} \right]$$

$$= P_K H_o \left[C - \left(1 + \frac{K_o}{H_o} \right) (H_i) \right].$$

Putting $1 + \frac{K_o}{H_o} = D$ (a constant) we have

$$\frac{dH_i}{dt} = P_K H_o [C - D(H_i)]$$

and putting $P_K(H_o) = V$ we have

$$V = \frac{1}{Dt} \ln_e \left[\frac{C}{C - D(H_i)} \right]$$

In case the external solution is relatively small the case is more complicated but if the permeabilities to the two ions are not very different, so that we may put as an approximation $P_K = P_H$ (where P_H is the permeability to H), we can simplify the treatment as the total quantity (not the net amount) of cations passing in any one direction, which we may call Q, will be approximately constant (since, other things being equal, the exchange of K^+ for H^+ will go on at approximately the same rate as the exchange of H^+ for H^+, etc., and in any case the total concentration of cations, both inside and outside

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We then have

$$\frac{dH_i}{dt} = P_K H_o \left[K_{ib} + H_{ib} - H_i - \frac{(H_i)(K_o)}{H_o} \right]$$

$$= P_K H_o \left[10 + 0 - 5 - \frac{(5)(50)}{50} \right]$$

$$= P_K H_o (0)$$

$$= 0$$

remains constant). The total quantity of H^+ passing inward in unit time will be

$$Q\left(\frac{H_o}{H_o + K_o}\right)$$

and that passing outward will be

$$Q\left(\frac{H_i}{H_i + K_i}\right)$$

and for the net amount passing in we may write

$$\frac{dH_i}{dt} = \frac{Q(H_o)}{H_o + K_o} - \frac{Q(H_i)}{H_i + K_i}$$

Since $H_o + K_o$ is a constant, we may call it A and in the same way put $H_i + K_i = B$, so that we have[10]

$$\frac{dH_i}{dt} = \frac{Q(H_o)}{A} - \frac{Q(H_i)}{B}$$

$$= \frac{Q}{AB}\left[(H_o)B - (H_i)A\right]$$

$$= \frac{Q}{A}\left[H_o - \frac{A}{B}(H_i)\right]$$

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[10] To illustrate numerically we may put $H_{ob} = 95$, $K_{ob} = 0$, $K_{ib} = 37.5$, $H_{ib} = 10$. When 25 H^+ has gone in and 25 K^+ has come out we have equilibrium for

$$\frac{A_o}{A_i} = \frac{95}{37.5 + 10} = 2, \frac{H_o}{H_i} = \frac{70}{35} = 2, \text{ and } \frac{K_o}{K_i} = \frac{25}{12.5} = 2$$

We then have

$$\frac{H_o + K_o}{H_i + K_i} = \frac{70 + 25}{35 + 12.5} = 2$$

so that

$$\frac{dH_i}{dt} = U_1\left[H_o - U_2(H_i)\right]$$

$$= U_1\left[70 - 2(35)\right]$$

$$= U_1(0)$$

$$= 0$$

Regarding Q as constant and putting

$$\frac{Q}{A} = U_1 \text{ and } \frac{A}{B} = U_2$$

we have

$$\frac{dH_i}{dt} = U_1 [H_o - U_2 (H_i)]$$

If the external solution is well buffered so that H_o is relatively constant we have

$$U_1 = \frac{1}{tU_2} \ln_e \frac{H_o}{H_o - U_2(H_i)}$$

When H_o is not constant the treatment is still comparatively simple. For convenience the net amount of H^+ which has entered at the time t is called x and it is assumed that the internal and external volumes are equal (*e.g.*, 1 liter). Using the subscript b to denote concentrations at the beginning we have the following:[11] $H_o = H_{ob} - x$; $K_i = K_{ib} - x$; $K_o = K_{ob} + x$, and $H_i = H_{ib} + x$. Substituting these values in the equation[12]

$$\frac{dx}{dt} = P_K [(H_o)(K_i) - (K_o)(H_i)]$$

we have

$$\frac{dx}{dt} = P_K [(H_{ob} - x)(K_{ib} - x) - (K_{ob} + x)(H_{ib} + x)]$$

$$= P_K [(H_{ob}K_{ib} - K_{ob}H_{ib}) - x(K_{ib} + H_{ob} + H_{ib} + K_{ob})]$$

---

[11] If the volumes are not equal another coefficient is needed. If, for example, the internal volume is half the external a gram mol of H^+ entering will have twice the concentration inside, so that if we put $x = 1$ we must put $H_i = H_{ib} + 2x$.

[12] Here P_K is (as before) the permeability to the slower ion K^+.

Putting $H_{ob}K_{ib} - K_{ob}H_{ib} = F$ and $K_{ib} + H_{ob} + H_{ib} + K_{ob} = G$ we have[13] (F and G being constants)

$$\frac{dx}{dt} = P_K(F - Gx)$$

$$P_K = \frac{1}{tG} \ln_e \frac{F}{F - Gx}$$

We thus arrive at a more general equation involving no assumptions except those made at the start.

All that is said regarding exchange of cations of course applies equally well to exchange of anions when the cations are indiffusible.

SUMMARY

Equations are given for a variety of cases when ions of the same sign are exchanged between the interior of a living cell and the external solution.

[13] To illustrate numerically we may put $H_{ob} = 95$, $K_{ob} = 0$, $K_{ib} = 37.5$, $H_{ib} = 10$. At equilibrium $x = 25$ and we have

$$\frac{dx}{dt} = P_K(F - Gx)$$

$$= P_K [H_{ob}K_{ib} - H_{ib}K_{ob} - x(K_{ib} + H_{ob} + H_{ib} + K_{ob})]$$

$$= P_K [(95)(37.5) - (10)(0) - 25(37.5 + 95 + 10 + 0)]$$

$$= P_K (0)$$

$$= 0$$

THE ACCUMULATION OF ELECTROLYTES

II. Suggestions as to the Nature of Accumulation in Valonia

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(Accepted for publication, August 1, 1930)

It is a familiar fact that electrolytes often appear to contravene physical laws by diffusing against a gradient[1] and attaining a higher concentration inside a living cell than outside.

This happens in the multinucleate cell of the marine alga *Valonia macrophysa*, consisting of a film of protoplasm (only a few microns in thickness) surrounding a large vacuole filled with clear watery sap (outside the protoplasm lies a cellulose wall, but it is so permeable to electrolytes that it need not be considered in this discussion).

Striking differences are seen on opposite sides of the protoplasmic film; for example we find only 0.012 M KCl in the sea water but there may be more than 40 times as much in the sap. We may therefore say that KCl accumulates[2] to a marked degree.

It is natural to enquire whether this can be accounted for by the Donnan principle, but Table I indicates that this is not the case.

[1] In some cases an ion may appear to go against a gradient when in reality this is not the case. It has been shown (Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255) that undissociated CO_2 comes rather quickly into equilibrium across the protoplasm. Let us suppose that owing to differences in pH value the dissociation in the sap is 20 per cent and in the sea water 80 per cent. If the concentration of undissociated CO_2 is the same inside and outside and is called 100, we have for the total inside 100 + 20 = 120, and outside 100 + 80 = 180. If the amount of sea water outside is small and the cell produces enough CO_2 to raise the undissociated CO_2 on each side to 200 we shall have (let us say) 30 HCO_3^- inside and 160 outside.

[2] The term "accumulation" is used to denote the process by which a penetrating substance reaches a greater concentration inside the cell than outside.

TABLE I*

Molar Concentrations

	Bermuda sea water	*Valonia macrophysa*
	M	M
Cl + Br	0.58	0.60
Na	0.5	0.09
K	0.012	0.5
Ca	0.012	0.0002?
Mg	0.057	Trace?
SO_4	0.036	Trace?
H	10^{-8}	$10^{-5.8}$
Organic matter, parts per thousand		1.433

* *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, **5**, 225 (where the value of Ca in the sap is erroneously given).

The Donnan principle requires the relation $K_i \div K_o = Na_i \div Na_o = H_i \div H_o = Cl_o \div Cl_i$ (where the subscripts *i* and *o* refer to inside and outside activities respectively) but these conditions are not fulfilled for according to Table I the observed values[3] are as follows:

$$K_i \div K_o = 0.5 \div 0.012 = 41.60$$

$$Na_i \div Na_o = 0.09 \div 0.5 = 0.18$$

$$H_i \div H_o = 10^{-5.8} \div 10^{-8} = 158.49$$

$$Cl_o \div Cl_i = 0.58 \div 0.6 = 0.97$$

Let us see whether we can employ the Donnan principle to explain the accumulation of K by making certain modifications. We might get rid of the necessity of putting $K_i \div K_o = Na_i \div Na_o$ by regarding Na as indiffusible since it enters more slowly than K but then we should still be unable to explain the facts for the concentration of indiffusible ions would not be high enough to account for the observed value of $K_i \div K_o$. Suppose we regard the Na of the sea water as indiffusible and neglect the counter-effect of the Na inside[4] (*i.e.*, suppose that we

[3] The values can only be approximate since they vary with external conditions so that the ratio of K + Na may be as low as 2.55 or as high as 5.72. For convenience we employ concentrations in place of activities.

[4] Neglecting the counter-effect of the Na inside (about 0.1 M) would more than compensate for neglecting the indiffusible cations outside, *i.e.*, the Mg in excess of SO_4 (about 0.057 − 0.036 = 0.021 M) and the Ca (about 0.012 M) and would therefore favor the attempt to use the Donnan principle more than would be strictly warranted by the facts.

have only KCl inside). We should then have $(K_o)(Cl_o) = (0.012)(0.58) = 0.007$ and $(K_i)(Cl_i) = (K_i)^2 = 0.007$ whence $K_i = 0.084$. This is far below the observed value of K_i (which is 0.5) and unless we should assume an impossible concentration of an indiffusible anion inside[5] we could not account for the observed value of K_i on the Donnan principle.[6]

The difficulty becomes still greater when we consider Cl for if KCl is diffusible we must put at equilibrium $K_i \div K_o = Cl_o \div Cl_i$. Evidently in order that *Valonia* may exist at all the osmotic pressure outside cannot be greater than inside so that Cl_o cannot be greater than Cl_i. If we say that Cl_o must be approximately equal to Cl_i then at equilibrium K_i must be approximately equal to Cl_o.

We escape these difficulties if we abandon the notion of equilibrium and say[6] that KOH and NaOH are constantly entering[7] the cell because their thermodynamic potential is greater outside than inside.

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[5] The internal concentration of anions other than Cl is extremely small.

[6] Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, **24**, 234; *Bulletin National Research Council*, No. 69, 1929, 170.

[7] The assumption is made later on (p. 294) that the entering KOH combines with a weak acid H*A* produced by the cell. This process may be regarded as practically equivalent to exchanging K^+ for H^+ if we consider only the thermodynamic situation which takes no account of how substances go through the protoplasm. For example (neglecting effects of hydrolysis) we may say that starting with 10 mols of H*A* inside and 2 of KOH outside and allowing 1 mol of KOH to enter there would be outside 1 of KOH and inside 1 of K*A* plus 9 of H*A*; this is equivalent to letting 1 equivalent of H^+ pass out in exchange for 1 of K^+ going in.

In the brief preliminary paper[6] this was not discussed because ionic exchange is regarded as relatively unimportant but it now requires mention since Brooks (Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1929, **27**, 75; *Protoplasma*, 1929, **8**, 389; and in Contributions to Marine Biology, Stanford University, California, 1930) states that as H^+ tends to pass out and anions cannot accompany it, K^+ comes in to maintain electrical neutrality. But then, in order to explain the behavior of Cl, it is assumed that HCO_3^- and Cl^- are diffusible, in direct contradiction to the former assumption. It is said that anions do not travel in the same paths as the cations but this really makes no difference since thermodynamically it does not matter how the ions get through. If H^+ and HCO_3^- pass out by different paths they can do so simultaneously and thus maintain electrical neutrality without intake of K^+ or Cl^-. That $H^+ + HCO_3^-$ can pass rapidly through the protoplasm (as such or in the form of neutral molecules) is shown by the work of M. M. Brooks (*Public Health Reports*, Washington, D. C., 1923, **38**, 1470).

The statement that HCO_3^- exists at a much higher concentration in the sap of *Valonia* than in the sea water is not borne out by the analyses of Dorcas (*cf.* Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255).

The thermodynamic potential of KOH is higher outside as long as the ionic activity product $(a_{K_o})(a_{OH_o})$ is greater than $(a_{K_i})(a_{OH_i})$. Assuming for convenience that the activity coefficients equal unity we may say that when the pH value of the sap is 5.8 and that of the sea water 8.0, we have $OH_i = 10^{-8.2}$ and $OH_o = 10^{-6}$ so that we may write as an approximation $(K_i)(OH_i) = (10^{-0.3})(10^{-8.2}) = 10^{-8.5}$ and $(K_o)(OH_o) = (10^{-2})(10^{-6}) = 10^{-8}$. In order to produce equilibrium we should have to raise the internal concentration of K to 1.585 M (so that $(K_i)(OH_i) = (10^{0.2})(10^{-8.2}) = 10^{-8}$) or raise the internal pH value to 6.3 (so that $(K_i)(OH_i) = (10^{-0.3})(10^{-7.7}) = 10^{-8}$).

Thus we can predict that if we raise the internal pH value higher than 6.3 K should begin to come out, and experiments carried out by Dr. Cooper and Mr. Jacques, in collaboration with the writer, show that this actually happens when the internal pH value is sufficiently raised by the entrance of NH_3.[8] This, of course, does not apply to NaOH since we have approximately $(Na_o)(OH_o) = (10^{-0.3})(10^{-6}) = 10^{-6.3}$ and to get equilibrium we should have to raise the internal concentration of Na to the impossible figure of 79.5 M (so that $(Na_i)(OH_i) = (10^{1.9})(10^{-8.2}) = 10^{-6.3}$) or raise the internal pH value to 8.7 (so that $(Na_i)(OH_i) = (10^{-1})(10^{-5.3}) = 10^{-6.3}$). Hence if the internal pH value is raised to the point where K begins to come out Na should continue to go in, as was actually found in the experiments just referred to.

Let us now consider the relative permeabilities to KOH and NaOH. If K^+ and OH^- penetrate by combining at the surface of the protoplasm to form molecules or enter as the ion pair $K^+ + OH^-$, as suggested elsewhere,[9] the quantity passing in will be proportional to the

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The hypothesis ignores the fact that the thermodynamic potential of KCl is much higher inside than outside, a matter of vital importance in connection with the inward movement of K which tends to maintain or increase this difference.

There seems to be no ground for assuming that at equilibrium H_i would be greatly in excess of H_o but if it were Cl_i would have to be greatly in excess of Cl_o and Cl would have to be regarded as indiffusible in order to explain the ratio of $H_i + H_o$.

[8] *Cf.* Cooper, Wm. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117.

[9] Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, **24**, 234; *Bulletin National Research Council*, No. 69, 1929, 170; *J. Gen. Physiol.*, 1929–30, **13**, 261.

number of collisions and hence to the ionic product[10] $(K_o)(OH_o)$: the quantity passing out will be proportional to $(K_i)(OH_i)$ and the net amount passing, or Q_{KOH}, will be proportional to the difference of ionic product[11] (which we may call p) so that we may put $p = B[(K_o)(OH_o) - (K_i)(OH_i)]$ where B is a proportionality factor (which may be omitted in comparing strong electrolytes in the subsequent discussion).

If we define the permeability to KOH (or P_{KOH}) as the amount passing through unit protoplasmic surface into the vacuole in unit time with unit difference of ionic product (*i.e.*, when $p_{KOH} = (K_o)(OH_o) - (K_i)(OH_i) = 1$) the amount passing in will be proportional to P, p, surface, and time, so that we may write[12]

$$Q_{KOH} = P_{KOH}\, p_{KOH} st \quad \text{and} \quad P_{KOH} = \frac{Q_{KOH}}{p_{KOH} st}$$

where s is surface, t is time, and Q is the number of gram mols passing in the time t. We may put as an approximation[13,14] $p_{KOH} = (K_o)(OH_o) - (K_i)(OH_i) = 0.012\ (10^{-6}) - 0.52\ (10^{-8.2}) = 87.2\ (10^{-10})$.

In the same way we have for NaOH the value $p_{NaOH} = (Na_o)(OH_o) - (Na_i)(OH_i) = 0.5\ (10^{-6}) - 0.09\ (10^{-8.2}) = 4994.3\ (10^{-10})$.

[10] It might seem that the amount entering would be proportional to the difference between the thermodynamic potential inside and outside or to

$$\frac{RT}{F} \ln [(a_{K_o})(a_{OH_o})] - \frac{RT}{F} \ln [(a_{K_i})(a_{OH_i})]$$

but this is not the case (*cf.* van Laar, J. J., Lehrbuch der theoretischen Elektrochemie, Wilhelm Engelmann, Leipsic, 1907, pp. 85 ff.).

[11] That p is analogous to an osmotic pressure gradient is evident from the following: If we assume that K^+ and OH^- colliding at the outer surface of the protoplasm form molecules of KOH the number produced in unit time will be proportional to $(K_o)(OH_o)$ and at the inner surface the number will be proportional to $(K_i)(OH_i)$. This also applies if we have to do with the formation of an ion pair $K^+ + OH^-$ (*i.e.*, K^+ and OH^- entering together).

[12] This resembles the formula of Northrop (Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435). See also Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

[13] The value is an approximation because we should use activities and also for other reasons. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

[14] The pH value of the sap is taken as 5.8.

Let us now consider the value of Q. If a cell is in vigorous growth, increasing 1 per cent a day,[15] the volume of the sap being 1 cc. and containing 0.52 M KCl and 0.09 M NaCl, the amount of K passing in per day may be regarded as $Q_{KOH} = 0.52\ (10^{-5})$ mol and in the same way $Q_{NaOH} = 0.09\ (10^{-5})$ mol.

We now have (if s and t are the same in both cases)

$$\frac{P_{KOH}}{P_{NaOH}} = \frac{Q_{KOH} \div p_{KOH}}{Q_{NaOH} \div p_{NaOH}} = \frac{0.52\ (10^{-5}) \div 87.2\ (10^{-10})}{0.09\ (10^{-5}) \div 4994.3\ (10^{-10})} = 331$$

In other words, the protoplasm appears to be about 331 times as permeable to KOH as to NaOH.[16]

Not much quantitative significance is to be attributed to these figures and in any case they can apply only where such factors as the movement of KCl, NaCl, and ionic exchange can be neglected.

This difference between KOH and NaOH is greater than would be expected if these substances entered in ionic form with the mobilities now assigned to them. Measurements[17] indicate that if we put the mobility of Cl^- in the outermost layer of protoplasm equal to 1, that of Na (or U_{Na}) is 0.2; in general, all the anions seem to have about the same mobility so that we may assume that of OH (or V_{OH}) to be equal to 1. The mobility of K (or U_K) is not yet accurately determined, but even if we put it as high[18] as 80 the figures do not correspond to those given above for we have (if we may use the same formula as for aqueous media) for D_{KOH}, the diffusion coefficient[19] of KOH in the outer layer of the protoplasm,

[15] This rate of growth is realized under favorable conditions.

[16] According to André and Demoussy (André, G., and Demoussy, E., *Bull. Soc. chim. biol.*, 1925, **7**, 806) K may diffuse more rapidly than Na in the aqueous media of the plant. Even if the permeability to K and Na were equal and the sap came into equilibrium with the solution bathing the cell, the greater diffusibility of K would make the fluid bathing the cells of certain parts of multicellular plants richer in K and would thus help to account for the preponderance of K in the sap of such cells.

[17] Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445.

[18] According to Damon (unpublished results) it is less than 80.

[19] *Cf.* Taylor, H. S., Treatise on physical chemistry, D. Van Nostrand Co., New York, 1924, p. 937.

$$D_{KOH} = RT\frac{2U_K V_{OH}}{U_K + V_{OH}} = RT\frac{2(80)(1)}{80+1} = 2RT$$

and for D_{NaOH}, the diffusion coefficient of NaOH,

$$D_{NaOH} = RT\frac{2U_{Na} V_{OH}}{U_{Na} + V_{OH}} = RT\frac{2(0.2)(1)}{0.2+1} = 0.33\,RT$$

Hence

$$\frac{D_{KOH}}{D_{NaOH}} = \frac{2}{0.33} = 6$$

According to this the permeability of the outer layer of the protoplasm to KOH is 6 times as great[20] as to NaOH but we have calculated earlier that of the protoplasm as a whole to be about 331 times as great. The calculation which gives the value 6 assumes the penetration of ions only; the other calculation applies whether molecules or ions penetrate but if ions alone penetrate it should agree with the

[20] If it were assumed (*cf.* Brooks, S. C., *Protoplasma*, 1928, **8**, 389) that H^+ inside is exchanged for K^+ outside and that K^+ does not tend to pass through the protoplasm more rapidly than H^+ we might calculate the permeability (P_K) to K^+ as follows: We put

$$P_{K^+} = \frac{Q_{K^+}}{p_{K^+} st}$$

where $p_{K^+} = (K_o)(H_i) - (K_i)(H_o)$, Q_{K^+} is the number of equivalents of K^+ passing in during the time t, s is surface, and P_{K^+} (the permeability to K^+) is the number of equivalents of K^+ entering in unit time when $s = 1$ and $p_{K^+} = 1$. We should then have approximately (putting $s = 1$ and $t = 1$)

$$\frac{P_{K^+}}{P_{Na^+}} = \frac{0.52\,(10^{-6}) \div (0.012)\,(10^{-6.8}) - (0.52)\,(10^{-6})}{0.09\,(10^{-6}) \div (0.5)\,(10^{-6.8}) - (0.09)\,(10^{-6})}$$

$$= 331$$

Since unpublished measurements indicate that U_K in the outer layer is not more than 80 times U_{Na} it might be necessary to assume a considerable difference between the concentration of K^+ and Na^+ in some part of the protoplasm to give the figure obtained by this calculation.

This calculation agrees with that given on p. 290 since thermodynamically the two cases are alike.

calculation which gives the value 6 and since it does not we may suppose that it is principally molecules that penetrate, or that concentration (and perhaps reaction) in the protoplasm plays a more important rôle than diffusion constants.

It has been shown elsewhere[21] that the mobilities of ions in the protoplasm do not follow the same order as in water. This (and other evidence) points to the fact that the protoplasmic surface is non-aqueous and it therefore seems quite probable that K^+ and OH^- combine at this surface to pass through as molecules just as H^+ and Cl^- combine at a water-air surface to pass through the air as molecules and dissociate again on reaching an aqueous medium. For if the surface of the protoplasm forms a non-aqueous layer the concentration of molecules of KOH in this layer will in all probability be much greater than that of the ions K^+ and OH^- so that, other things being equal, the permeability to molecules will be much greater than the permeability to ions.

For the same reason it does not seem probable that exchange of ions[22,23] plays a great rôle though the cell can of course produce H^+, NH_4^+, and organic anions which are available for exchange.[24]

This point of view is in harmony with the fact that the effective resistance of the protoplasm is high.[25]

[21] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715.

[22] In the experiments with NH_3 the amount of K coming out does not correspond to the amount of NH_4 appearing inside and the process does not appear to be ionic exchange (Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301).

[23] For the kinetics of ionic exchange see Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 277.

[24] Earlier results with *Nitella* indicating slow ionic penetration were probably due largely to reversible injury (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1921–22, **4**, 275).

For a discussion of ionic exchange in *Valonia* see Höber, R., and Höber, J., *Arch. ges. Physiol.*, 1928, **219**, 260. If the entrance depends on ionic radius (in an aqueous medium) as postulated by Höber, Cs should go in more rapidly than K but this is not the case (*cf.* Cooper, Wm. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928–29, **12**, 427).

See also Brooks, S. C., *Protoplasma*, 1929, **8**, 389.

CORRECTION

In foot-note 25, on pages 292–293, Vol. **14**, No. 2, November, 1930, the first line should read: "The effective resistance is $\frac{E}{I}$; the ohmic resistance." The last line of this foot-note, reading "The effective conductance is $1 \div R$," should be omitted.

The idea that there is little or no penetration of ions is supported by experiments with CO_2. It is found[26] that if the concentration of undissociated molecules of CO_2 or H_2CO_3 is kept constant the rate of penetration remains constant even though great variations occur in the concentration of ions. The behavior of the time curves at high and low pH values leads to a similar conclusion.[27]

In this paper we shall for convenience speak of the penetration of electrolytes in molecular form without implying that ionic penetration does not occur but only that it must play a subordinate rôle owing to the non-aqueous character of the protoplasmic surfaces.

These remarks do not affect anything that is said concerning equilibrium since this is in no way dependent on the mode of entrance. But it should be remembered that in a living and growing cell no equilibrium is possible, the nearest approach to it being the sort of pseudo-equilibrium observed with certain rapidly penetrating substances[28] such as CO_2 and H_2S.

We may sum up by saying that penetration in ionic form would seem to be much slower than in molecular form. If penetration occurs by means of ion pairs (*e.g.*, as $K^+ + OH^-$) we should have the equations already given but this would not be the case if it occurs by exchange, *e.g.*, if K^+ enters in exchange for H^+ the amount passing through[29] would be proportional to $(K_o)(H_i) - (K_i)(H_o)$.

Let us now consider the distribution of anions. Evidently the

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the current (whether an applied potential or one existing in the plant or a combination of both), and P is the polarization or back E.M.F. produced by the plant during the passage of the current (opposing the passage of the current). In most cases P accounts for most of the observed resistance.

The effective conductance is $1 + R$.

[26] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 695.

[27] It is often assumed that if a weak acid penetrates more rapidly at low pH values it must enter chiefly in molecular form but this is not justified since, for example, lowering the pH value increases undissociated CO_2 or H_2CO_3, and the product $(H)(HCO_3)$ in the same proportion (since $(H)(HCO_3) = C(H_2CO_3)$ where C is the dissociation constant). See Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

[28] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255; Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–28, **8**, 131.

[29] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 277.

penetration of KOH and NaOH would raise the internal pH value if the cell were not continually producing acids (*e.g.*, carbonic and other organic acids).[6] If we lump these acids under the term HA[30] we may say that the entering KOH will produce $K^+ + A^-$ thus increasing the concentration of A^- if HA is a weak acid, as is probably the case. As this process continues A^- will increase and will tend to pass out of the cell[31] either as A^- or as undissociated HA which will dissociate on reaching the sea water. If, for example, we imagine a cell having as the only solute in the sap KA at a concentration of 0.6 M (with a small amount of undissociated HA) there will be a tendency for A^- to go out and for Cl^- to move in. This could be effected by exchange of A^- for Cl^-, passing through the protoplasmic surfaces as such or as undissociated HA and HCl (which dissociate again after traversing the surface). In the absence of complications this might go on until A_i became equal to A_o.

When Donnan constraints are present the exchange will tend to produce a condition where $A_i \div Cl_i = A_o \div Cl_o$. Such constraints are present in *Valonia*[32] for we have in the sea water indiffusible ions such as Mg^{++} and $SO_4^=$ (the latter in small concentration), which are normally not found in the sap,[33] and Ca^{++} which, if present at all in the sap, is found only in traces. We also have Na^+ which enters so slowly that it may be regarded as practically indiffusible in comparison with ions which enter rapidly. If HCl and HA^- (or Cl^- and A^-) exchange promptly we might expect them to distribute themselves without much delay according to the Donnan principle so that in the absence of complications there would be a tendency to make $A_i \div Cl_i = A_o \div Cl_o$.

When the exchange has raised the value of $(K_i)(Cl_i)$ to the point where it is equal to $(K_o)(Cl_o)$, *i.e.*, to (0.012) (0.58) = 0.007, the difference of thermodynamic potential of KCl may be regarded as

[30] The concentration of HA will presumably be small owing to the fact that it is constantly leaving the cell.

[31] A^- will tend to pass out when $(H_i)(A_i)$ is greater than $(H_o)(A_o)$ or $(K_i)(A_i)$ is greater than $(K_o)(A_o)$ even though A_i be less than A_o (1).

[32] They do not bring the system into Donnan equilibrium because the continual production of acid and other metabolic activities prevent it.

[33] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, **5**, 225.

approximately zero and any further exchange of HA for HCl (or of Cl^- for A^-) will increase the difference of thermodynamic potential of KCl and lessen that of KA (since the value of A_o, owing to the large external volume, must be very small and in consequence the product $(K_o)(A_o)$ must be nearly zero). How far the energy of the latter process is available for increasing the difference of thermodynamic potential of KCl is not certain but in any case there must be an abundant supply of energy for this purpose in the metabolism of the cell. This will be discussed in subsequent papers when additional data are presented.

Since K is found in the sap in combination with Cl it is evident that if it really enters as KOH and reacts to form KA there must be an exchange of A^- for Cl^- or of HA for HCl.

We may sum up by saying that under normal conditions the difference of thermodynamic potential of KOH tends to drive K in[34] and that of KCl tends to drive K out: if the latter be greater[35] metabolic energy would be required to drive K in and this case may have some relation to that described by Straub.[36]

Turning to a consideration of Cl we may say that the difference of thermodynamic potential of KCl tends to drive Cl out and that of NaCl tends to drive Cl in and that these forces seem to be not far from equal.[37]

It may be noted that the production of HA and the exchange of a weak acid for HCl tend to depress the pH value of the sap; on the

[34] The entrance of KOH would temporarily disturb the existing approximate osmotic equilibrium. This might be compared to the well known experiment in which a closed porous pot containing air and fitted with a manometer is placed in an atmosphere of hydrogen. The entrance of hydrogen is more rapid than the exit of air and in consequence the pressure rises temporarily in the pot. The bursting of cells in dilute alcohol may be due to a similar cause.

[35] The difference of thermodynamic potential of KCl is approximately proportional to $(K_i)(Cl_i) - (K_o)(Cl_o) = (0.5)(0.6) - (0.01)(0.58) = 0.2942$, and that of KOH to $(K_o)(OH_o) - (K_i)(OH_i) = (10^{-2})(10^{-6}) - (10^{-0.3})(10^{-8.2}) = 0.684\,(10^{-9})$.

[36] Straub, J., *Rec. grav. chim. Pays-Bas*, 1929, **48**, 49.

[37] The difference of thermodynamic potential of KCl is approximately proportional to $(K_i)(Cl_i) - (K_o)(Cl_o) = (0.5)(0.6) - (0.01)(0.58) = 0.2942$, and that of NaCl to $(Na_o)(Cl_o) - (Na_i)(Cl_i) = (0.5)(0.58) - (0.1)(0.6) = 0.23$.

other hand the entrance of KOH and NaOH tends to raise it. A balance appears to be struck between these antagonistic processes so that the pH value of the sap does not vary widely and this (as suggested elsewhere[36]) may be due to a regulatory process.

We might therefore, as an alternative hypothesis, regard H_i as approximately constant and since H_o is constant we could say that the ratio $H_i \div H_o$ is fixed and therefore produces a constant potential difference[38] across the protoplasm so that all the other ions (whether passing through in ionic or molecular form) will strive to conform to it[39] so that $K_i \div K_o$ and $Na_i \div Na_o$ will tend to equal $H_i \div H_o$.[40] But this would involve the necessity of making $Cl_o \div Cl_i$ tend toward the same value which is evidently not possible if the cell is to exist with practically no anion except Cl^- inside.

It should be emphasized that the normal ratio of $H_i \div H_o$ (which is far from equilibrium) is maintained at a high value (about 160) by the energy of metabolism which continually produces HA and which maintains the ratio of $K_i \div K_o$ and $Cl_i \div Cl_o$. It is quite possible

[38] This would be small compared with that due to K since the concentration of H is so small, for a change in the pH value of the sea water produces little change in the P.D. across the protoplasm.

[39] The fixed ratio $H_i \div H_o$ implies a P.D. across the protoplasm which would act on all ions present.

[40] This is in harmony with what has preceded. If, for example, we say that KOH tends to enter until $(K_o)(OH_o) = (K_i)(OH_i)$ it is equivalent to saying that K tends to enter until $K_i + H_i = K_o + H_o$ since if we put

$$OH_o = \frac{K_w}{H_o} \quad \text{and} \quad OH_i = \frac{K_w}{H_i}$$

we have

$$(K_o)\left(\frac{K_w}{H_o}\right) = (K_i)\left(\frac{K_w}{H_i}\right)$$

whence

$$\frac{K_o}{H_o} = \frac{K_i}{H_i}$$

Hence if the external solution remains constant and the internal pH value is raised so that H_i is decreased we should expect K to come out.

that when HA is once produced the transfer of molecules and ions, *e.g.*, exchange of HA for HCl or of A^- for Cl^-, follows without further expenditure of energy.

As another alternative it may be suggested that as HA and KOH are present in such small amounts they may be neglected and KCl may be considered to move in as such. But this scheme would not present so clearly the rôle of the pH value of the sap which, as will be shown in subsequent papers, is very important.

Let us now consider how we may picture the process of penetration and growth. The production of a weak acid HA inside the cell adds to the existing osmotic pressure which is further increased by the entrance of KOH and NaOH, transforming HA to its salts, and under normal conditions water will enter until the internal pressure nearly equals that outside, as the cell wall is too elastic to offer much resistance.

The entrance of water will dilute any electrolytes which have already reached a pseudo-equilibrium with the outside solution and will therefore favor an inward[41] movement.

With the growth of the cell proceeding in this manner the total concentration of electrolytes inside should be approximately constant as appears to be actually the case.

It seems probable that the rate of penetration will be influenced by the activity of the cell (*e.g.*, by production of acid, or of substances which increase osmotic pressure and absorption of water (such as sugars) or of those which promote the expansion of the cell wall (such as carbohydrates); possibly also substances may be formed which directly influence permeability.

Should the activity of the cell (production of acid, etc.) decline the tendency will be to approach a true Donnan equilibrium, with the same ratio of K to Na inside and outside.[42] In consequence, the ratio $K_i \div Na_i$ will fall off and this is actually observed when activity of the cell is lowered by cooling.

The explanation given here is merely a working hypothesis which must be tested experimentally. Many tests have been made during the last five years which support the hypothesis and which will be

[41] The dilution of Cl inside may thus favor its entrance in exchange for A^-.

[42] Death leads to a different condition with the inside and outside exactly the same, and injury tends to produce this condition.

discussed in subsequent papers. For the present we may confine our attention to a consideration of experiments with NH_3 such as have recently been described.[43]

When NH_4Cl is added to the sea water it soon makes its appearance inside where its concentration may become many times as great as outside. According to our hypothesis we should expect undissociated molecules of NH_3 or NH_4OH to enter more rapidly than ions and this is evidently the case since the pH value of the sap rises rapidly, apparently because the penetration of NH_3 or NH_4OH is more rapid than the production of acid in the cell: this accords with the fact (shown by analysis) that the total NH_3 (by which is meant NH_3 + undissociated NH_4OH + NH_4) enters the cell much more rapidly than is normally the case with K or Na.

According to our hypothesis the entering NH_3 or NH_4OH combines with HA to form NH_4A and at first there is little exchange but after a few minutes HA begins to be exchanged for Cl^- so that there should be a steady increase of NH_4Cl in the sap. This is found to be the case; as a result of this exchange and of the production of acid by the cell the pH value of the sap soon stops rising since the result of the exchange is to replace the weak acid HA by the strong acid HCl; if this went far enough it would cause the internal pH value to fall off again after its original rise (when the cell is first put into sea water containing NH_4Cl) and such a falling off is commonly observed.[44]

The rise in pH value will affect the entrance of KOH and NaOH. The penetration of KOH should begin to fall off much sooner than that of NaOH and as the pH value of the sap continues to rise K should begin to come out while Na continues to enter. This is because the ionic activity products differ as explained on p. 288. The experiments show that this prediction is correct.[44]

[43] Cooper, Wm. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117; Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301; in these experiments the cells lived indefinitely and grew better than when no NH_3 was added.

[44] The rate at which this occurs depends in fact on the rate of entrance of NH_3 (or NH_4OH) and that of the exit of KOH. It should be remembered that when the pH value of the sap is raised CO_2 may move inward from the sea water since the CO_2 equilibrium will be disturbed.

If we now consider effects upon growth we see that even if the rise in pH value due to NH_3 does not stimulate the production of acid by the cell the rapid entrance of NH_3 will cause a quicker rise in osmotic pressure than is normally the case. The result will be an increased entrance of water and distension of the cellulose wall. Before NH_3 is added the entrance of KOH and NaOH and consequent absorption of water seems to be slow enough to permit the cell wall to stretch and keep pace with the entrance of electrolytes and in consequence the internal concentration of Cl remains relatively steady. But NH_3 (or NH_4OH) enters much more rapidly than K or Na so that it seems possible that the cell wall cannot stretch rapidly enough to keep up and in consequence the total concentration of electrolyte should increase somewhat. This is apparently the case. At first the total concentration increases from about 0.6 M to about 0.61 M after which a balance appears to be struck between the pressure and the elasticity of the cell wall so that the total concentration of electrolyte becomes relatively constant.

This increase of pressure explains in part at least the more rapid growth which we observe when NH_4Cl is present in the sea water. It is also quite possible that NH_3 contributes to this by increasing permeability.

It is evident from all that has been said that we can predict the behavior of K better by disregarding the movement of KCl and paying attention to that of KOH. It may be remarked incidentally that such evidence as we have[44] (which is not conclusive) indicates that the permeability to KCl is not greater than to NaCl and that in consequence the movement of KCl may be neglected.

The experiments with NH_4Cl seem therefore to support the hypothesis devised to explain what occurs under normal conditions. This hypothesis may be summarized by saying that the energy necessary to produce the accumulation of K is furnished by metabolism in connection with the production of a weak organic acid H*A* produced in the cell, and H*A* is exchanged for HCl of the external solution (or *A* for Cl) so that it is very much as if the cell produced HCl continually and maintained an internal pH value of 5.8 (that of the sea water being about 8). Under these circumstances KOH diffuses in to neutralize the acid inside. This tends toward a condition in which

the product (K)(OH) is the same inside and outside so that if the internal pH value remains 5.8 K_i must become greater than K_o. If KCl is formed in the sap faster than it comes out there must be an accumulation and this is understandable if KCl passes chiefly in molecular form and permeability to molecules of KCl is low.

This is not an equilibrium condition for if the expenditure of energy stops and acid is no longer produced in the cell the internal pH value will fall and K will come out so that eventually the distribution of electrolytes inside and outside will tend to become nearly equal.[45]

It may be added that experiments on artificial models show that when a difference of pH value is maintained on opposite sides of a partition an electrolyte may pass over from the alkaline to the acid side and accumulate to a marked extent even though no indiffusible ions are present. These experiments will be described in subsequent papers.

SUMMARY

It is suggested that K enters chiefly as KOH, whose thermodynamic potential (proportional to the ionic activity product (a_K) (a_{OH})) is greater outside than within. As this difference is maintained by the production of acid in the cell K continues to enter, and reaches a greater concentration inside than outside. KOH combines with a weak organic acid which is exchanged for HCl entering from the sea water (or its anion is exchanged for Cl^-), so that KCl accumulates in the sap.

Na enters more slowly and its internal concentration remains below that of K.

The facts indicate that penetration is chiefly in molecular form.

As the system is not in equilibrium the suggestion is not susceptible of thermodynamic proof but it is useful in predicting the behavior of K, Na, and NH_4.

[45] The excess of Mg^{++} over $SO_4^{=}$ in the sea water would tend to produce a Donnan equilibrium.

THE ACCUMULATION OF ELECTROLYTES

III. Behavior of Sodium, Potassium, and Ammonium in Valonia

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(Accepted for publication, August 1, 1930)

As stated[1] elsewhere, we should theoretically expect that when NH_3 enters the cell and raises the pH value of the sap beyond a certain point K may come out while Na continues to go in.

Experiments carried out by Dr. Cooper and the senior author[2] showed that the entrance of NH_3 was accompanied by the exit of K but the behavior of Na was left uncertain. It was not realized at that time that the K ÷ Na ratio in the sap could be so easily affected by external conditions, *e.g.*, by handling the cells or by changing the sea water or by illumination. But in the present experiments conclusive results have been secured by controlling the conditions more carefully, *e.g.*, by keeping the cells continuously in running sea water,[3] avoiding the handling during the experiment and taking care that in each experiment all the cells were equally illuminated.

The effect of light is important since a moderate amount of illumination (but not direct sunlight) promotes growth.[2] For example, when cells were kept side by side in ordinary sea water in the open but not in direct sunlight, one lot in a glass container gained 13 per cent in 20 days but those in a similar container which was painted black outside made no gain.[4] Under natural conditions the cells grow in clumps several layers thick and the illumination therefore varies from

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 285.

[2] Cooper, Wm. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117. For effects of light on absorption in *Nitella* see Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, **6**, 47.

[3] Brooks, S. C., (*Protoplasma*, 1929, **8**, 389) states that the K ÷ Na ratio is increased by increase or decrease of the normal amount of K in the sea water.

[4] The difference in temperature in the two tubes was probably small.

layer to layer, so that we should anticipate marked variations in a group of cells collected at one time and place.

To this variation in the natural state we must add the possibility of changes brought about by handling. Blinks[5] has shown that in *Valonia ventricosa* and *Nitella* the direct current resistance is reduced to a very low value by mechanical shock, and that even handling is sufficient to produce marked effects which may last a surprisingly long time. All this suggests the necessity for reducing the handling to the minimum, and allowing a period of rest after collection in order to get rid of the effects of pulling the clumps apart.

The first step in our work was the separation of the cells (immediately after collection); this produces a slight softening of most of the cells, and subsequently about 10 per cent die. Fortunately it is possible in Bermuda to get cells free from sponges and algae, so that the problem of cleaning is not troublesome.

TABLE I

	K	Average	Difference	Na	Average	Difference
	M		*per cent*	M		*per cent*
A	0.4902 0.4897	0.4900	0.1	0.1289 0.1296	0.1293	0.6
B	0.4986 0.5004	0.4995	0.4	0.1210 0.1200	0.1205	0.8
C Large	0.4743	0.4927	7.8	0.1378	0.1258	22.0
C Small	0.5111			0.1138		

After separation the cells were stored out of doors (not in direct sunlight) in 3-gallon glass bottles. At intervals the sea water was changed, the cells being disturbed as little as possible during this operation. The sea water was changed daily during the first few days after collection, in order to avoid undue contamination by potassium-rich sap from injured cells, and dead cells were removed as discovered. Subsequently the change was made every 3 to 5 days. The cells were stored under these conditions for at least a month (in some experiments much longer).

The object of this seasoning process was to produce a uniform group of cells. This point was tested in the following way. Cells which had been collected and treated as described above, were divided into 3 groups of the approximate ranges, 0.75–0.6 cc., 0.6–0.4 cc., and 0.4–0.2 cc. Three selections, *A*, *B*, and *C*, were then made, each containing 5 of the large, 15 of the medium, and 20 of the small cells. Selections *A* and *B* were then extracted: each yielded about 12 cc. of sap,

[5] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361, 495.

enough for analysis in duplicate for K and Na in each sample. Selection *C* was divided into two parts, the one containing enough large and medium cells for a single determination of K and Na, and the other comprising the remainder (small cells). The results are given in Table I (which shows that with lots *A* and *B* the difference between duplicate analyses was less than 1 per cent but that the difference between large and small cells is much greater, indicating that the K ÷ Na ratio is higher in small cells, *i.e.*, K ÷ Na is 3.44 for large and 4.49 for small cells, as would theoretically be expected[1] if the small cells, being younger, are in a more active state). It therefore seemed best to use for each experiment a typical lot of cells, *i.e.*, a lot graded as to size in definite manner, arbitrarily called typical so that any typical lot will be comparable with any other.

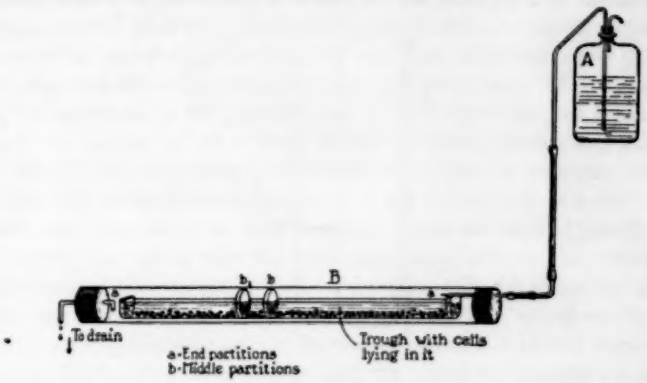

FIG. 1. Tube containing cells exposed to a solution constantly flowing through the tube from the reservoir *A*. The cells lie in a glass trough with perforated end walls (*a*) and perforated partitions (*b*).

In each experiment two typical lots of cells, alike in number and size, were exposed under exactly similar conditions to (*a*) sea water, and (*b*) sea water containing a small concentration of NH_4Cl. A certain number of cells in each group were selected for determination of volume, since it is not desirable to subject all the cells to the handling necessary for such measurements. At intervals cells were withdrawn from each group and the following determinations were made: K, Na, $NH_3 + NH_4$, total halide, and pH value. At the same time the changes in volume were measured. The total length of exposure was usually 30 days. The concentration of the NH_4Cl in the sea water ranged from 0.0025 to 0.001 M.

The work was carried out in Bermuda during the spring of 1930. During the period the lowest temperature recorded was 16°C. and the highest 26°C., the average temperature was 21°C. The procedure and results for a typical experiment are described in detail below.

A group of 650 cells were selected and divided into two groups, the first con-

sisting of a typical lot, to be used for volume measurement, and the second containing the remainder (also a typical lot). The cells were placed in a pyrex glass trough, which was essentially a half glass tube of about 1 inch radius. This trough was blocked at both ends with paraffin wax partitions, perforated to allow the sea water, but not the cells, to pass through, with two similar partitions (*b* and b_1, Fig. 1) cutting off a portion of the trough near the center. In this portion the volume control cells were exposed, while the other cells occupied the portions on either side. This arrangement was designed to give the volume cells an exposure similar to the average exposure of the other cells. The trough, 34 inches long, was placed in a pyrex tube of 2¼ inches diameter, 36 inches long. The tube was fitted with one-hole rubber stoppers, through one of which was thrust a tube drawn down to a capillary at the end and connected by rubber and glass

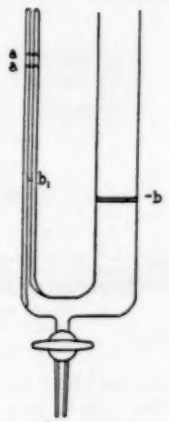

FIG. 2. Apparatus for determining the volume of living cells (explanation in text).

tubing to a reservoir of sea water plus NH_4Cl, and a flow of fresh solution was maintained through the tube as long as the experiment lasted. The other rubber stopper carried a short syphon tube to serve as a drain (Fig. 1). As a control an exactly similar selection of cells was exposed to sea water in another apparatus, under the same conditions, without the addition of NH_4Cl. Both tubes were placed side by side out of doors (but not in direct sunlight) and fed with fresh solution at the rate of about 20 drops per minute, a rate sufficiently rapid to prevent serious changes in concentration but not great enough to disturb the cells mechanically.

At the start of the experiment a typical lot of 100 cells, was taken and after a rapid rinse in distilled water, the cells were dried by rolling on filter paper, and the sap was extracted by piercing the cellulose wall with a fine glass capillary and squeezing the cell. The determinations mentioned above were then made, *i.e.*,

K, Na, NH_3 + NH_4, total halide and pH value. This set of determinations, called hereafter the initial values, serves as the reference point to which all subsequent determinations are referred.

The initial volumes of the two typical lots of cells were next determined in the apparatus shown in Fig. 2. The volume tube was a U-tube with one wide arm and a capillary arm of about 1.5 mm. radius. A stopcock at the bottom was added for convenience in calibrating and cleaning the apparatus. The apparatus was held by a ring stand clamped to a solid table—and readings were made by means of a 1-meter brass scale cathetometer, reading to 0.005 cm. (made by Gaertner). This also was fixed in position.

In making a determination, the volume apparatus was first partially filled with sea water and inserted in its clamps. All the volume cells for the particular determination on hand were then poured out on nichrome gauze, allowed to drain for a moment, and then transferred one at a time to several layers of filter paper where they were allowed to drain for from 30 to 60 seconds. During this time the level of the meniscus in the capillary arm of the U-tube was read, and the distance between two marks "*a*" as a check of the mechanical operation of the cathetometer. Finally the cross hairs were set on one of the "*a*" reference marks. The cells were then dropped in the wide arm of the U-tube, one at a time. When all the cells had been added we first ascertained that the level of the apparatus had not changed (by noting that the cross hairs were still on the reference mark), and determined the change in level in the wide tube by observing the distance that the meniscus had risen in the capillary arm. There was of course a capillary effect but as both the wide and capillary arms of the U-tube had been selected for uniformity it is reasonable to assume that this would be the same for all readings. To make it possible to transform the cathetometer readings to cubic centimeters the tube was calibrated by weighing the distilled water corresponding to changes in the meniscus level for several intervals.

In measuring the volume of the cells the errors involved are, the error of setting the cathetometer, error due to difficulty in determining the bottom point of the meniscus, error due to lack of uniformity in the cross sections of the arms of the U-tubes, and the error due to incomplete drainage of the cells. The first of these errors can be practically eliminated by carefully levelling the telescope at each reading. The second was made negligible by reading the meniscus in the narrow arm where its width is so small that the lowest point can be located with ease. The third error was made small by using in the construction of the apparatus tubes which had been gauged for uniformity. The last error is the most important. It is not desirable to wipe the cells dry with cloth or filter paper, since this procedure has been found to result in the death of a fairly large proportion of them. We have, therefore, to depend on drainage on filter paper to dry them. We have estimated the total error to be expected from all the causes above by making three complete measurements of the same group, wetting them thoroughly between measurements. The measured volumes were: 6.015, 6.040, 6.020, giving

an average of 6.025, a maximum deviation from the mean of 0.42 per cent and an average deviation from the mean of 0.17 per cent. These deviations are negligible compared with the deviations of the analyses due to lack of uniformity of the cells.

During the course of a volume measurement, a cell occasionally died. We allowed for this by measuring the volume of the dead cell at the time of death, and the volume of all the cells, and in subsequent measurements we added the former to the found volume of the remaining cells. It is clear that when this procedure becomes necessary, the measured volume plus the correction for any subsequent point in a run will be too small by an amount equal to growth which the dead cell would have experienced from the time of death until the time of the measurement in question, had it not died. No doubt a correction to the correction could have been estimated, based on the growth of the whole group for any period. But the advisability of attempting this in view of the magnitude of other errors in the work seemed questionable. In the detailed experiment, in fact no volume cells were lost so that error from this cause does not enter. It is estimated that it did not in any case exceed 0.5 per cent.

The analyses for K and Na were made in the following manner: The sap was centrifugalized in covered tubes for 20 minutes to remove chloroplasts and as much as possible of the jelly-like clots which are always present in the expressed sap. Two 5-cc. samples were then taken and evaporated to dryness on the water bath, dried in the oven and finally ignited at 300° to 350°C. to remove all traces of carbon, and in the case of the ammonia group the NH_4Cl also. The residue was weighed and the assumption was made that it was a mixture of NaCl and KCl only. Occasionally a sample of sap was found to have a trace of SO_4 on testing with acidified $BaCl_2$, and at one time our procedure involved the removal of this and of any possible traces of Mg and Ca before evaporation. A comparison of the results obtained after removal with those obtained without removal suggested that this extra labor was not worth while.

The K was determined as $KClO_4$ in the usual way, and the Na was calculated by difference from the weights of the total chlorides and the weight of KCl corresponding to the weight of K-perchlorate found.

A determination of the accuracy of our method of analysis made on a known mixture of K-, Na- and NH_4-chlorides resembling sap of cells after exposure, gave the following results:

	Found	*Actual*	*Per cent deviation*
K	0.5013	0.5000	0.26
Na	0.0982	0.1000	1.8

These results were obtained at the outset of the work when the analytical procedures were unfamiliar, and subsequent results are doubtless better. Nevertheless, since we could use conveniently only 5 cc. of sap for each analysis the weight of sodium chloride is small, in general about 0.04 gram, and this coupled

with necessity for making the weighing of the combined salts very rapidly, in order to prevent the absorption of weighable amounts of water in the very moist atmosphere of Bermuda, throws a considerable burden on the accuracy of the Na analysis.

The halide was determined by Mohr titration with $AgNO_3$, on a duplicate pair of 1-cc. samples.

The pH value was determined in a single 1-cc. sample as a rule, but occasionally the sap of a single cell was compared against a like volume of buffer solution. Chlorophenol red was the indicator used.

A single analysis for ammonia was made on the sap of the cells before exposure. It was found that the amount present was less than could be determined satisfactorily by our usual method. A trial later on other unexposed cells, using the much more delicate Nessler's reagent, indicated that there is less than 0.0005 mols per liter normally present in cells which have been stored in changes of sea water obtained far enough from land to be free of contamination.

The usual method of determining ammonia was distillation of the free ammonia into a known amount of standard acid, and determination of the amount used up by titration of the unused acid with standard CO_2-free alkali using methyl red as indicator. An estimation of the accuracy of the method in our hands was made by determining ammonia on a known sample containing 0.0500 mol of ammonium chloride per liter. The results are:

	Found	*Actual*	*Per cent deviation*
NH_4	0.0500	0.0510	2.0

(Duplicate 1-cc. samples were used for ammonia determination.)

Tables II and III and Fig. 3 show the results of an experiment in which sufficient 0.6 M NH_4Cl was added to bring the concentration of NH_4Cl in the sea water up to 0.001 M.

If we suppose, as stated elsewhere,[1] that KOH enters,[6] and assume, for convenience, that the activity coefficients equal unity[7] we may say as an approximation that equilibrium will be reached when $(K_o)(OH_o) = (K_i)(OH_i)$, where the subscripts i and o refer to the activities (concentrations) inside and outside. Under normal conditions (when the pH value of the sea water is 8 and that of the sap 5.9) we have $(K_o)(OH_o) = (10^{-2})(10^{-6}) = 10^{-8}$. In order to produce equilibrium

[6] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

[7] This makes practically no change in the present case where only relative activities and concentrations are considered, *i.e.*, where different activities or concentrations are compared.

TABLE II

Changes in the Sap of Valonia Produced by Exposure to Sea Water at pH 8 Containing 0.001 M NH_4Cl

Days	Control group						Ammonia group						
	Molecular concentration				Volume	pH	Molecular concentration					Volume	pH
	K	Na	Total* cations	Halide			K	Na	$NH_3 + NH_4$	Total* cations	Halide		
					cc.							*cc.*	
0	0.4922	0.1338	0.6260	0.6092	5.450	5.9	0.4922	0.1338	0.0005	0.6260	0.6092	5.125	5.9
2	0.4905	0.1276	0.6181	0.6070	5.570	5.9	0.4714	0.1345	0.0166	0.6225	0.6170	5.265	6.4
5	0.4916	0.1317	0.6233	0.6088	5.635	5.9	0.4557	0.1468	0.0297	0.6322	0.6183	5.400	6.0
7	0.4980	0.1245	0.6225	0.6164	5.660	5.9	0.4328	0.1481	0.0390	0.6199	0.6190	5.555	6.2
10	0.4909	0.1266	0.6175	0.6158	5.665	5.9	0.4160	0.1551	0.0592	0.6303	0.6164	5.675	6.0
15	0.4902	0.1269	0.6171	0.6183	5.670	5.9	0.3963	0.1581	0.0801	0.6345	0.6208	5.800	6.2
20	0.4921	0.1252	0.6173	0.6149	5.685	5.9	0.3675	0.1469	0.1119	0.6263	0.6202	5.900	6.2
30	0.4906	0.1299	0.6205	**	5.720	5.9	0.3579	0.1497	0.1252	0.6328	**	5.950	6.0

* The excess of total cations over halide has been discussed in previous work (*Cf.* Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, **7**, 633).

** Analyses made on single samples and halide omitted owing to insufficiency of cells.

we should have to raise the pH value of the sap to 6.3 (so that $(K_i)(OH_i) - (10^{-0.3})(10^{-7.7}) = 10^{-8}$). In other words, if we raise the pH value of the sap above 6.3 we should expect K to come out because the thermodynamic potential inside, which is proportional to $(K_i)(OH_i)$, would be greater than outside.

Fig. 3 shows that K comes out and Table II shows that the pH value of the sap has risen[2] as the result of penetration of NH_3 to about 6.3, but this value is probably too low as previous determinations[2] gave 6.6. It may be said that the determinations of pH value are

TABLE III

Gram Mols in Sap

Days	Gram mols (10^3) = concentration × volume in cc.							
	Control			Ammonia group				
	K	Na	Halide	Corrected volumes	K	Na	$NH_3 + NH_4$	Halide
0	2.683	0.7293	3.320	5.450	2.683	0.7293	0.00273	3.320
2	2.733	0.7108	3.382	5.599	2.640	0.7530	0.09294	3.456
5	2.770	0.7422	3.430	5.743	2.617	0.8430	0.17057	3.550
7	2.818	0.7407	3.490	5.907	2.555	0.8750	0.23050	3.656
10	2.782	0.7161	3.488	6.036	2.510	0.9360	0.35630	3.720
15	2.780	0.7197	3.507	6.167	2.448	0.9750	0.49400	3.828
20	2.797	0.7117	3.497	6.275	2.305	0.9218	0.70220	3.891
30	2.806	0.7430	—	6.327	2.260	0.9472	0.79220	—

not very accurate owing to the high concentration of salt and the fact that the sap is practically unbuffered.

Let us now consider the behavior of Na. The external ionic product is $(Na_o)(OH_o) = (10^{-0.3})(10^{-6}) = 10^{-6.3}$ which is greater than the internal or $(Na_i)(OH_i) = (10^{-0.57})(10^{-7.7}) = 10^{-8.57}$ even when the pH value of the sap is 6.3 (*i.e.*, when $OH_i = 10^{-7.7}$). Hence we should expect Na to go in. As a matter of fact Na does continue to penetrate and at a greater rate than before.[8] This might be attributed to injury but as a matter of fact these cells lived indefinitely and grew better than cells not exposed to ammonia and microscopic examination

[8] It happens that these controls show very slight gain in mols of Na in 30 days but in other experiments there was more.

showed nothing abnormal. It might, however, be thought that a few injured cells which allowed Na to enter and K to come out were responsible for the result. This seems improbable in view of the

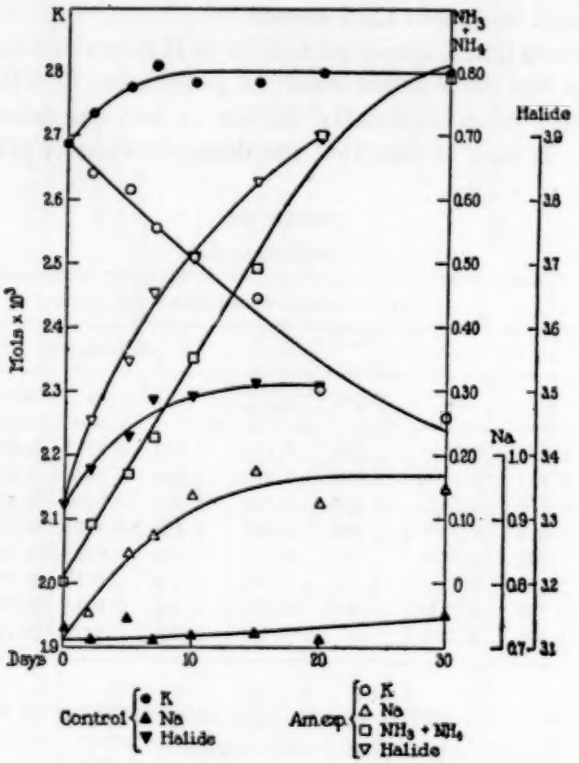

FIG. 3. Graphs showing the change in mols of K, Na, $NH_3 + NH_4$, and halide in a typical lot of cells in sea water (control) and in an equivalent lot of cells in sea water containing 0.001 M NH_4Cl (the scales of ordinates for each substance are the same but are displaced vertically to bring the curves into one figure). The lowest curve is drawn with the results of other experiments in mind.

The data represent a single typical experiment, using about 50 cells for each determination.

fact that if there were enough injured cells to produce this result a much greater mortality would be expected than was actually found and moreover the amount of SO_4 in the samples of sap analyzed was

not above the normal. It may be added that similar results have been gotten repeatedly in other experiments which would imply the same degree of injury in all cells exposed to ammonia (if the result were attributed to injury).

The rate of penetration of Na would tend to rise above the normal due to the dilution of electrolytes inside caused by the entrance of water (which is more rapid than normal as shown by the increased rate of growth) but on the other hand the rise in the pH value of the sap would tend to depress the rate since it would decrease the driving force (which is proportional to the difference between the external and internal thermodynamic potential).

The increased rate of entrance of Na may be due to an increase in permeability caused by NH_3 and this would harmonize with the fact that the exit of K is more rapid than would be expected from its normal rate of penetration. We note that (0.002806 − 0.002683 =) 0.000123 mol of K entered the controls but in the same period the exit of K from the cells exposed to ammonia amounted to (0.002683 − 0.002260 =) 0.000423 or more than 3 times as much. Before concluding that this is due to increase in permeability we must consider the ionic products. If we neglect the effect of the cell wall (as seems justified in the case of *Valonia macrophysa* under normal conditions, on account of the great elasticity of the wall) we may put,[1] as stated elsewhere,

$$P_{KOH} = \frac{Q_{KOH}}{p_{KOH} st}$$

where P_{KOH} is the permeability[9] to KOH, Q is the amount passing in during the time t, and s is surface. For the entrance of K during 30 days the value of Q is 0.000123 mol, and for comparative purposes we may put $s = 1$ and $t = 1$. The value[10] of p is taken as $(K_o)(OH_o) - (K_i)(OH_i) = (10^{-2})(10^{-6}) - (10^{-0.3})(10^{-8.1}) = 6\ (10^{-9})$. We then have

$$P_{KOH} = \frac{0.000123}{6(10^{-9})} = 20500$$

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[9] *I.e.*, the amount passing through the protoplasm in unit time, through unit surface with unit value of p (*i.e.*, when $(K_o)\ (OH_o) - (K_i)\ (OH_i) = 1$).

[10] This is an approximation, since strictly speaking we should employ activities, and for other reasons. Round numbers are here employed.

In the same way for the exit of K, when the pH value of the sap is taken as 6.6 and the concentration of K in the sap is given for convenience an average value for the whole period of 0.43 M, the value of p_{KOH} is $(K_i)(OH_i) - (K_o)(OH_o) = (10^{-0.37})(10^{-7.4}) - (10^{-2})(10^{-6}) = 0.7\,(10^{-8})$. Hence for the exit of K we have (neglecting any difference in s)

$$P_{KOH} = \frac{0.000423}{0.7(10^{-8})} = 60429$$

According to this the permeability during the exit[11] exceeds the permeability during the entrance by (60429 ÷ 20500) = 2.9 times. These figures indicate a decided increase in permeability but their quantitative significance is not very great as some of the data are uncertain[12] and, moreover, a part of the increase might be due to enlargement of the surface.

It might be suggested that the rapid exit of K is due to an exchange of K^+ for NH_4^+ but if the entrance of NH_4^+ depended on such exchange we should not find the rate of entrance increasing with the external pH value[13] as is actually observed.[2] We note also that the amount of ammonia entering is considerably less than the amount of K coming out: for example, in the first two days 0.0208 mol of K has come out and only 0.0161 mol of NH_4 has gone in.

An exchange of K^+ for Na^+ might be assumed but this would not be a rapid process.

Moreover the internal pH value would not rise (as it is seen to do) if only NH_4 and not NH_3 or NH_4OH went in; nor would the total halide increase.

It may be of interest to calculate permeability to NaOH going in

[11] It must be remembered that the entering K would be increasing osmotic pressure (*e.g.*, by transforming a weak organic acid in the cell to its salt) with consequent opposition due to stretching the cell wall: the exit would not encounter this opposition which, however, in the present case seems to be negligible.

[12] In the case of Na the data for the control are too uncertain to make a calculation worth while.

[13] It might be argued that the increase in external pH value would increase the negativity of the protoplasmic surface and thus favor the exchange of cations, but this would be counterbalanced by the falling off in the number of NH_4 ions.

as compared with that to KOH coming out. We have (taking the figures for 7 days and using average values for K_i and Na_i during this period)

$$\frac{P_{KOH}}{P_{NaOH}} = \frac{Q_{KOH\ outward} \div \not{p}_{KOH\ outward}}{Q_{NaOH\ inward} \div \not{p}_{NaOH\ inward}}$$

$$= \frac{0.000128 \div [(0.463)(10^{-7.4}) - (10^{-8})(10^{-6})]}{0.0001457 \div [(0.5)(10^{-6}) - (0.141)(10^{-7.4})]}$$

$$= 51.7$$

This is much less than the value of 331 calculated elsewhere for normal conditions[1] when both KOH and NaOH are entering.

Under normal conditions we cannot calculate permeability to KCl since the internal ionic product is greater than the external but in this case if we suppose KCl to pass out we can compare the value of P_{KCl} going out with P_{NaCl} coming in. We have

$$\frac{P_{KCl}}{P_{NaCl}} = \frac{Q_{KCl\ outward} \div \not{p}_{KCl\ outward}}{Q_{NaCl\ inward} \div \not{p}_{NaCl\ inward}}$$

$$= \frac{0.000128 \div [(0.463)(0.618) - (0.01)(0.58)]}{0.0001457 \div [(0.5)(0.58) - (0.141)(0.618)]}$$

$$= 0.64$$

This has not much quantitative significance since it involves the assumption that the per cent of Na moving in as NaCl is the same as the per cent of K moving out as KCl.

A similar calculation for the figures at the end of 30 days gives $P_{KCl} \div P_{NaCl} = 1.5$. Hence if the calculations mean anything they signify that in the experiments with ammonia permeability to KCl is about the same as to NaCl.

In considering the increase in permeability due to the presence of ammonia we may recall Loeb's[14] suggestion that the protoplasmic surface may resemble a soap whose viscosity depends on the cation (thus NH_4 makes a softer soap than K, also K a softer soap than Na, and Na a softer soap than Ca).

It is also possible that the presence of one substance in the surface layer of the protoplasm may affect the solubility of other substances.

[14] Loeb, J., The dynamics of living matter, The Macmillan Co., New York, 1906.

It may be desirable to add a word regarding the increased growth in the presence of ammonia.[2] Theoretically this would be expected[1] if we suppose that NH_3 or NH_4OH on entering combines with a weak organic acid HA changing it to $NH_4 + A^-$ and so increasing osmotic pressure. This would also happen with the entrance of KOH under normal conditions (in absence of ammonia) but much more slowly for it is evident that the entrance of KOH is much slower than that of ammonia.

It is also possible that ammonia aids growth in other ways, *e.g.*, by increasing the production of acid in the cell or of substances which promote the expansion of the cell wall or increase permeability.

In view of the more rapid increase of osmotic pressure due to the entrance of ammonia it would not be surprising if the cell wall failed to stretch rapidly enough to keep up so that the total electrolyte inside would increase. This seems to happen[2] as shown in Table II.

The fact that the pH value does not rise beyond a certain point (*cf.* Irwin,[15] Brooks[16]) may be due to the production of acid on the part of the cell and this might possibly take place in a regulatory manner.

As stated elsewhere[1] we assume that there is an exchange of HA for HCl (or of A^- for Cl^-) of the sea water so that there is an accumulation of NH_4Cl: this would aid in keeping down the pH value as it is in effect the exchange of a weak for a strong acid.

SUMMARY

When 0.001 M NH_4Cl is added to sea water containing *Valonia macrophysa* there seems to be a rapid penetration of undissociated NH_3 (or NH_4OH) which raises the pH value of the sap so that the thermodynamic potential of KOH becomes greater inside than outside and in consequence K leaves the cell: NaOH continues to go in because its thermodynamic potential is greater outside than inside.

NH_4Cl accumulates, reaching a much higher concentration inside than outside. This might be explained on the ground that NH_3, after entering, combines with a weak organic acid produced in the cell whose anion is exchanged for the Cl^- of the sea water, or (more probably) the organic acid is exchanged for HCl.

[15] Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 235.

[16] Brooks, M. M., *Pub. Health Reports*, Washington, D. C., 1923, **38**, 2074.

MONOGRAPHS—*Continued*

No. 2. William Travis Howard and Oscar T. Schultz. Studies in the histogy of tumor cells. Plates 1–6. (Issued February 15, 1911.) Price, $1.00.

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